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(54) Title: SCREEN FOR THE IDENTIFICATION OF AGENTS USEFUL IN THE TREATMENT OF CANCERS

(57) Abstract: A method for identifying a substance which inhibits or prevents resistance to a chemotherapeutic agent or a radiotherapeutic agent and/or which may be used in the treatment of a cancer, which method comprises determining whether a test substance is an inhibitor of Grp78. Inhibitors of a nitric oxide synthase, inhibitors of Grp78 and substances identified in a method of the invention are useful in therapy.

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SCREEN FOR THE IDENTIFICATION OF AGENTS USEFUL IN THE TREATMENT OF CANCERS

Field of the Invention

This invention relates to methods for screening for new therapeutic agents. The therapeutic agents identified in the screen are particularly useful in cancer therapy. The invention also relates to an antibody which may be used in cancer therapy.

Background to the Invention

Nitric oxide (NO) is a pleiotropic signal molecule which has been identified as a mediator for a wide range of physiological and pathophysiological events. The diverse cellular signalling properties of NO are in part, due to redox-sensitive interactions with metal and thiol containing proteins. One major downstream target is the enzyme soluble guanylyl cyclase (sGC). NO binding to the heme domain of sGC results in a 200-400 fold increase in enzymatic activity, leading to an increase in the concentration of the intracellular messenger molecule cGMP.

It has also been shown that NO can mediate some of its biological effects through mechanisms involving the transcriptional regulation of a number of molecules including the $p21^{WAF1}$ cyclin-dependent kinase inhibitor and, in the presence of calcium, c-fos and c-jun. In addition, NO can also increase p53 protein concentration. This results in an increase in p53 stability and is likely to have effects on the transcription of p53-regulated genes. NO can also transcriptionally regulate the expression of the vascular endothelial growth factor, VEGF.

Summary of the Invention

In order to examine the role of NO on gene expression we have used a differential display protocol, involving the differential hybridization of mRNA-derived probes to normalized cDNA arrays.

Using this approach, we have shown that the generation of NO in a cell increases the expression of the hypoxia stress-induced endoplasmic reticulum chaperone Glucose-regulated protein 78 (Grp78) and results in cellular resistance to the apoptotic agent thapsigargin.

These results are highly significant in the context of cancer therapy. It seems

that NO may play a pathophysiological role in promoting tumor growth and in protecting tumors from agents that cause cell damage. NO production in cancer cells may thus confer resistance to chemotherapeutic agents.

Our findings open up a totally new strategy for cancer therapy, suggesting that administration of an inhibitor of NOS or an inhibitor of Grp78 can be used in combination with a chemotherapeutic agent or a radiotherapeutic agent to sensitize NO-producing tumor cells that would otherwise be resistant to apoptotic killing mechanisms. Screening for inhibitors of Grp78 should allow substances to be identified which will be useful in therapy, particularly in cancer therapy.

In view of these results, we decided to target the surface-localized Grp78 protein using single-chain antibody variable fragments (scFv) and to determine if such antibodies possessed cytotoxic and/or cytostatic properties. Panning of human phage display scFv libraries against recombinant Grp78 followed by further rounds of screening against whole cells resulted in the isolation of novel anti-Grp78 scFv. Exposure of breast and prostate cancer cells to anti-Grp78 scFv results in profound inhibition of growth. Furthermore, the anti-Grp78 scFv can attenuate the Grp78-mediated protection against thapsigargin (TG), a selective ER Ca^{2+}-ATPase inhibitor.

Taken as a whole, these data suggest that Grp78 should be considered as a new target for cancer diagnosis and therapy.

According to the present invention there is thus provided a method for identifying a substance which inhibits or prevents resistance to a chemotherapeutic agent or a radiotherapeutic agent and/or which may be used in the treatment of a cancer, which method comprises determining whether a test substance is an inhibitor of Grp78.

The invention also provides:

- an inhibitor of Grp78 for use in a method of treatment of the human or animal body by therapy;
- use of an inhibitor of Grp78 in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent;
- use of an inhibitor of a NOS in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent;

- use of an inhibitor of a Grp78 in the manufacture of a medicament for use in the treatment of a cancer;
- a method of treating or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host, which method comprises the step of administering to the host an effective amount of an inhibitor of Grp78 or an inhibitor of a NOS;
- a method of treating a cancer in a host, which method comprises the step of administering to the host an effective amount of an inhibitor of Grp78;
- a substance identified by a method of the invention for identifying a substance which inhibits or prevents resistance to a chemotherapeutic agent or a radiotherapeutic agent and/or which may be used in the treatment of a cancer;
- a substance of the invention for use in a method of treatment of the human or animal body by therapy;
- use of a substance of the invention in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of a cancer;
- a method of treating or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host, which method comprises the step of administering to the host an effective amount of a substance of the invention;
- a method of a cancer in a host, which method comprises the step of administering to the host an effective amount of a substance of the invention;
- products containing an inhibitor of a NOS and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer;
- products containing an inhibitor of Grp78 and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer;
- products containing a substance of the invention and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer;
- use of an inhibitor of a NOS in the manufacture of a medicament for use with a

chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer;

- use of an inhibitor of Grp78 in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer;
- use of a substance of the invention in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer;
- a method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of an inhibitor of NOS and a chemotherapeutic agent or a radiotherapeutic agent;
- a method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of an inhibitor of Grp78 and a chemotherapeutic agent or a radiotherapeutic agent;
- a method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of a substance of the invention and a chemotherapeutic agent or a radiotherapeutic agent.;
- a method for determining the presence of a cancer in a subject, which method comprises typing the gene encoding Grp78 in the subject; and
- an scFv having the sequence set out in SEQ ID NO: 2 or a functional variant thereof.

Brief description of the Drawings

Figure 1 shows increase of Grp78 expression by NO. **a** Gene expression profiles in uninduced and induced (10 μM muristerone A, 16h) EcR293 clone 11 subclone-1 cells. On well D12, Grp78 expression is 3-fold higher in induced cells as compared with uninduced cells. **b**. Northern blot analysis of Grp78 mRNA from either tetracycline-induced Tex293 clone 22 cells uninduced (Lane 1) and induced (Lane 2 - labelled tet-22) or muristerone A treated EcR293 clone 11 subclone-1 cells uninduced (Lane 3) and induced EcR293 clone11 subclone-1 cells (Lane 4). **c**. Scanning densitometry was used to normalize Grp78 mRNA levels relative to those of β-actin in uninduced and induced cells. Values are means (±SD) from three separate experiments. **d**. Western blot analysis of Grp78 protein from the uninduced cells and induced cells in the presence of tetracycline or muristerone A. α-tubulin was used as a control. **e**. Western blot analysis of Grp78 protein in Tex293 cells treated with the NO donor

DETA-NONOate. **f.** NO-dependent increase in Grp78 promoter activity. Upper panel, the Grp78 promoter region, which contains 3 endoplasmic reticulum stress response elements (ERSE) fused with a luciferase reporter gene in the vector (pGL3 basic). 48 h after transient transfection of the construct into Tex293 cells, the cells were treated with different doses of DETA NONOate. The relative luciferase activities were calculated as described in Song *et al.* (Cancer Res. 15, 8322-8330, 2001) after co-transfection of the promoter-reporter plasmid and the internal control plasmid pRL-tk.

Figure 2 shows NO-mediated cytoprotection against thapsigargin (TG)-mediated toxicity. **a.** The Griess reaction was used to measure NO in the medium of EcR293 clone 11 subclone-1 cells MuA = 10 μM muristerone A, MuA/L-NIO = 10 μM muristerone A plus 20 μM L-NIO. **b.** Cytotoxicity of TG was determined by LDH in muristerone A-induced and uninduced cell cultures. Values are the means (±SD) of three replicates. **P < 0.01 indicates a significant difference between uninduced and induced cells. **c.** Grp78 antisense oligonucleotides attenuates the NO-mediated cytoprotection against TG toxicity. EcR293 clone 11 subclone-1 cells were induced to generate NO by treatment with muristerone A (10 μM) for 16 h. Either 20 μM Grp78 antisense, or Grp78 sense oligonucleotides were added during the induction time. The cell culture then treated with 37 ng/ml TG, and TG-mediated cytotoxicity was determined by LDH induced after 48 h treatment. Values are the means (± SD) of three replicates. **P < 0.01 indicates a significant difference between uninduced and induced cells. **d.** CMV promoter driven human Grp78cDNA construct, pLP-Grp78 (see GenBank accession no. M19645 [gi:183644] for the human Grp78 nucleic acid and amino acid sequences), was transfected in EcR293 cell and selected with G418 to establish a permanent cell line, which expresses 3-4-fold more Grp78 protein than the empty vector control (upper panel). The cell culture then treated with 19ng/ml of TG and cytotoxicity of TG was determined by LDH after 48 hrs treatment. Values are the means (± SD) of three replicates. **P < 0.01 indicates a significant difference between Grp78cDNA transfected cell and the control empty vector transfected cells.

Figure 3 shows that Grp78 mRNA is over-expressed in human beast cancer cells. Total RNA was isolated from paired tumor (T) and adjacent normal tissue (N) and subjected to northern blotting with a Grp78 cDNA probe and with a human iNOS probe. Scanning densitometry was used to normalize Grp78 mRNA levels relative to those of 28S RNA (Ethidium bromide staining). The relative fold-increase is given in

comparison between tumor samples and normal samples for both Grp78 and iNOS.

Figure 4 shows that cytokine induction of NO, or exogenous administration of an NO-donor in breast cancer cell lines up-regulates Grp78. **a.** human breast cancer cell lines (MCF-7 and ZR75-1) were induced by cytokine cocktail mixture (CCM) for 24 h. The Griess reaction was used to measure NO in the medium of 10^6 cells in the presence or absence of NOS inhibitor, L-NIO. **b.** mRNA was isolated from both cell lines and subjected to northern blotting with a Grp78cDNA probe. β-actin was used as a control. **c.** Scanning densitometry was used to normalize Grp78 mRNA levels relative to those of β-actin. Relative fold-increase is given in comparison between CCM induced or uninduced cell cultures. **d.** Exogenous administration of an NO donor, (DETA NONOate) to breast cancer cell line ZR75-1. mRNA was subjected to northern blotting with a Grp78cDNA probe. β-actin was used as a control probe. **e.** Scanning densitometry was used to normalize Grp78 mRNA levels relative to those of β-actin. Relative fold-increase is given in comparison of untreated and treated cells with different doses of DETA NONOate.

f. Western blot analysis of Grp78 protein induction following treatment of the MCF-7 tet-on Grp78 cell line with 10 ng/ml of doxycycline. α-tubulin was used as a control. **g.** Treatment of breast cancer cell lines MCF-7 and ZR75-1 with antisense-Grp78 oligonucleotides (20 mM) results in increased cytotoxicity of TG as determined by the LDH assay. Values are the means (± SD) of three replicates. $^{**}P < 0.01$ indicates a significant difference between untreated and treated with sense oligonucleotides.

Figure 5 shows the effect of anti-Grp78 scFv on cell viability. **A.** 10 μg of Grp78-scFv clone-19 was added to the culture medium of EcR293 clone-11 subclone-1 cells to determine if the antibody could reverse the NO mediated cytoprotection against TG. Control cells were treated with 10 μg of scFv-20 (a negative control scFv that does not recognize Grp78). Treatment of the cells for 48 h resulted in a significant (more than 50%) reduction in cell survival in TG-treated NO-generating cells, while treatment with the same concentration of control antibody had no significant effect on cell viability. **B.** Apoptosis of prostate cancer PC3 cells (following staining with Hoechst 33342). PC3 cells alone in the absence of any further treatment exhibited very little apoptosis. Treatment with either TG or Grp78-scFv clone-19 showed increased apoptosis. The combination of Grp78-scFv clone-19 treatment and TG showed the most cell death.

Figure 6 shows that the anti-Grp78 scFv-19 has a direct effect on cell growth. Three cell lines (ZR75-1, PC3 and HB3) cells were treated with 50 µg/ml of Ni-purified soluble scFv-19. The growth rate was determined using a Beckman Coulter (Z2 series) Counter. Non-Grp78-reactive scFv-20 was used as a control. The panels show that the growth of both the breast cancer cell line ZR75-1 (**A**) and the prostate cancer cell line PC3 (**C**) is significantly retarded following treatment with the anti-Grp78 scFv-19, while the growth of the non-breast cancer luminal cell line HB3 (**B**) is not affected.

Figure 7 shows the nucleotide and deduced amino acid sequences for Grp78-scFv clone-19. The complementarity determining regions are shown underlined.

Brief description of the sequence listing

SEQ ID NO:1 sets out the nucleic acid sequence of the scFv of the invention.

SEQ ID NO: 2 sets out the amino acid sequence of the scFv of the invention.

Detailed Description of the Invention

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

We have been examining the biological consequences arising from the expression of NOS activity in a cell. NOS activity has been reported in both cancer cell lines and in tumour biopsies, and while high concentrations of NO appear to have anti-tumor properties, lower concentrations are thought to promote tumour growth and neovascularisation. Exposure of a cell to NO has been shown to alter the expression of the cellular transcriptome, and in order to study this process systematically we have developed two regulated cell expression systems where an inducible NOS (iNOS) cDNA is placed under the control of either a tetracycline or an ecdysone-inducible promoter. Following transfection, the resulting cell lines can generate NO in a dose- and time-dependent manner at concentrations found in biopsies of human tumours.

Using DNA-microarrays, we have shown that NO-generation in a cell results in the up-regulation of genes important in the protection of cells from environmental

stress. These include the DNA-dependent protein-kinase catalytic subunit (DNA-PKcs) and the glucose-regulated protein (Grp78). The sequences of various Grp78 genes (and their products) are readily available to those skilled in the art. For example, human Grp78 has the GenBank accession number M19645 [gi:183644].

Reference to Grp78 herein encompasses all Grp78 genes (and the associated gene products), including that having the accession number set out above (also see Ting and Lee, Human gene encoding the 78,000-dalton glucose-regulated protein and its pseudogene: structure, conservation, and regulation, DNA 7 (4), 275-286, 1988) and also non-human variants found in other species, for example the corresponding gene and gene product in a non-human animal such as a mammal.

Tumour cells treated with chemotherapeutic agents or radiotherapeutic agents often become resistant to the toxicity of that agent. We have identified an important principle underlying the molecular mechanism which leads to the evocation of resistance to toxicity.

We have found that increased NO generation by cells results in increased expression of the stress-induced endoplasmic reticulum chaperone Grp78 in those cells. It is that increase in Grp78 expression that confers resistance to the toxicity of certain chemotherapeutic agents and radiotherapeutic agents. This phenomenon may be referred to conveniently as NO-mediated Grp78 resistance to stress. This protection extends to the toxic effects of agents such as thapsigargin (TG).

These findings have led us to make the hypothesis that exposure of cells to sub-lethal 'priming' with NO may result not only in protection against higher concentrations of NO but also in protection from other cytotoxic agents, including reagents used therapeutically in the treatment of cancer.

The link we have established between increased NO generation, increased Grp78 expression and resistance to chemotherapeutic agents and radiotherapeutic agents presents a new target for the identification of substances useful in methods of treatment of the human or animal body by therapy.

To test this approach, we have targeted Grp78 as this protein is up-regulated by NO and is located on the surface of tumor cells making it accessible to antibodies. This has lead to the identification of scFv which targets cell surface localized Grp78. We have shown that NO-generating cells are resistance to thapsigargin by a process involving Grp78. However, when cells are treated with an anti-Grp78 scFv, there is a

significant reduction in cell survival indicating that the antibody represents a therapeutic approach for the treatment of cancers (perhaps in combination with thapsigargin or analogs thereof) in situations where Grp78 is over-expressed.

The invention thus provides a screening method for identifying a substance which inhibits the resistance of a cell, typically a cancer/tumor cell, to the toxicity of a chemotherapeutic agent or a radiotherapeutic agent. Such a method may be used in the identification of a substance which may be used in the treatment of a cancer. A method of the invention comprises determining whether a test substance is capable of inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent. Substances identified in this way may be used in the treatment of a cancer. In particular, a method of the invention comprises determining whether a test substance is an inhibitor of Grp78. Alternatively, a method of the invention may comprise determining whether a test substance is an inhibitor of a NOS.

The skilled person may thereby determine whether or not the test substance is one which inhibits the resistance of a cell, typically a cancer/tumor cell, to the toxicity of a chemotherapeutic agent or a radiotherapeutic agent and/or one which may be used in the treatment of a cancer.

A substance identified by a method of the invention, an inhibitor of Grp78 or an inhibitor of a NOS can be used in a method of treatment of the human or animal body by therapy. Typically such substances will be used in a method of inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent or in a method of treatment of a cancer. Those substances may therefore be used to treat resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host or to treat a cancer.

A substance identified by a method of the invention, an inhibitor of Grp78 or an inhibitor of a NOS may be used in the treatment of a cancer. The host may be a human or animal host.

A substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 may be used in combination with a chemotherapeutic agent or a radiotherapeutic agent in a method of treatment of the human or animal body by therapy. Thus, the invention provides products containing an inhibitor of a NOS, an inhibitor of Grp78 or a substance identified by a method of the invention and a chemotherapeutic agent or a radiotherapeutic agent may thus be used as a combined

preparation for simultaneous, separate or sequential use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent. In particular, combinational therapy will be useful in a method of treatment of a cancer.

The phrase "inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent" encompasses situations where a reduction in the level of an existing resistance to a chemotherapeutic agent or a radiotherapeutic agent is achieved. The phrase also encompasses the prevention of resistance to a chemotherapeutic agent or a radiotherapeutic agent resistance, i.e. the use of an inhibitor of a NOS, an inhibitor of Grp78 or a substance identified by a method of the invention to prevent resistance to a chemotherapeutic agent or a therapeutic agent developing in the first place or to reduce the amount to which resistance to a chemotherapeutic agent or a radiotherapeutic agent develops. That is to say, combinatorial treatment using a chemotherapeutic agent or a radiotherapeutic agent and a substance identified by a method of the invention, an inhibitor of Grp78 or an inhibitor of a NOS, reduces the degree to which resistance to a chemotherapeutic agent or a radiotherapeutic agent develops compared to treatment with that chemotherapeutic agent or that radiotherapeutic agent alone.

The invention provides a method for identifying a substance which inhibits cell resistance to the toxicity of a chemotherapeutic agent or a radiotherapeutic agent or which may be used in the treatment of a cancer. Typically, such a method of the invention comprises determining whether a test substance is an inhibitor of Grp78. However, a method of the invention may additionally or alternatively comprise determining whether a test substance is an inhibitor of a NOS.

An inhibitor of a Grp78 is a substance which inhibits expression, for example transcription and/or translation, of a Grp78 gene and/or inhibits activity of the Grp78 polypeptide.

An inhibitor of a NOS is a substance which inhibits expression, for example transcription and/or translation, of an NOS gene and/or inhibits activity of a NOS enzyme. An inhibitor of a NOS may be an inhibitor of eNOS, nNOS or iNOS. Preferably, the inhibitor will be a specific inhibitor of a particular NOS isoform. A specific inhibitor of a NOS isoform is one that inhibits the NOS isoform for which it is specific to a significantly greater degree than the other isoforms. Typically it will inhibit the isoform for which it is specific at least 2x as much as it inhibits any other isoform, for example at least 5x as much as any other isoform, in particular at least 10x

as much as any other isoform. Preferably, the specific inhibitor will inhibit the NOS isoform for which it is specific, but will have substantially no inhibitory effect on the other NOS isoforms.

Inhibition in this context indicates a reduction in Grp78/NOS activity in relation to the level of activity observed in the absence of an inhibitor.

Clearly, an inhibitor identified by a method of the invention, an inhibitor of GRP78 or an inhibitor of a NOS may inhibit transcription and/or translation of the gene against which it acts and/or activity of the polypeptide against which it acts. An inhibitor of the invention may act by binding to the relevant polypeptide. Ultimately, however, the cumulative overall effect will be important, as the polypeptide is typically the active species. Thus, when a substance inhibits transcription or translation, the effective "activity" of the corresponding polypeptide will be inhibited. Although the activity per mole of polypeptide will be unaltered; the amount of polypeptide will be simply diminished.

Typically, an inhibitor suitable for use in the invention will be specific for Grp78, which is to say that it will inhibit Grp78 to a substantially greater degree than any other gene or gene product. In particular, a specific inhibitor will inhibit Grp78 to a degree of about 2 to 1000 times, for example about 10 to 500 times, in particular about 50 to 100 times that to which it inhibits any other gene or gene product. For example, a Grp78 inhibitor may be one which inhibits Grp78, but which substantially does not inhibit any other gene or gene product.

A Grp78 (or NOS) inhibitor suitable for use in the invention may act by binding directly to the promoter of a gene encoding Grp78/NOS, thus preventing the initiation of transcription. Alternatively, an inhibitor could bind to a Grp78/NOS polypeptide/polypeptide complex which is associated with the promoter and is required for transcription. This may result in reduced levels of transcription.

An inhibitor may also inhibit expression of Grp78/NOS by binding directly to the untranslated region of an mRNA of a gene encoding Grp78/NOS. This may prevent the initiation of translation. Alternatively, an inhibitor may bind to a polypeptide/polypeptide complex associated with the untranslated region and prevent that polypeptide/polypeptide associating with the untranslated region.

An inhibitor of the activity of the Grp78/NOS gene product pathway (i.e. an inhibitor which acts on the enzymatic activity of the polypeptide encoded a Grp78/NOS

gene) may do so by binding to such a Grp78/NOS. Such inhibition may be reversible or irreversible. An irreversible inhibitor dissociates very slowly from its target because it becomes very tightly bound to the enzyme, either covalently or non-covalently. Reversible inhibition, in contrast with irreversible inhibition, is characterised by a rapid dissociation of the target-inhibitor complex.

The inhibitor may be a competitive inhibitor. In competitive inhibition, the Grp78/NOS can bind substrate (forming a Grp78/NOS-substrate complex) or inhibitor (Grp78/NOS-inhibitor complex) but not both. Many competitive inhibitors resemble the substrate and bind the active site of the Grp78/NOS. The substrate is therefore prevented from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of Grp78/NOS molecules bound to a substrate.

The inhibitor may be a non-competitive inhibitor. In non-competitive inhibition, which is also reversible, the inhibitor and substrate can bind simultaneously to a Grp78/NOS molecule. This means that their binding sites do not overlap. A non-competitive inhibitor acts by decreasing the turnover number of Grp78/NOS rather than by diminishing the proportion of Grp78/NOS molecules that are bound to substrate.

The inhibitor can be a mixed inhibitor. Mixed inhibition occurs when an inhibitor affects both the binding of substrate and alters the turnover number of the Grp78.

An inhibitor of a Grp78/NOS may act by binding to the substrate of such a Grp78/NOS. The substance may itself catalyze a reaction of the substrate, so that the substrate is not available to the Grp78/NOS. Alternatively, the inhibitor may simply prevent the substrate binding to the Grp78/NOS.

Suitable inhibitors may be antibody products (for example, monoclonal or polyclonal antibodies, single chain antibodies, chimaeric antibodies, CDR-grafted or humanised antibodies) which are, for example, specific for a Grp78. Antibody fragments may be used, including Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies. In addition, suitable inhibitors may be the products of so-called phage-display libraries, where recombinant phage is used to express single-chain fragments (scFv) of diverse antibody complexity. In this latter case, heavy and light chain variable regions are joined together using a flexible linker.

Suitable antibodies may be raised by standard techniques and are specific for a

polypeptide of the invention. Such antibodies could for example, be useful in purification, isolation or screening methods involving immunoprecipitation techniques, may be used as tools to further elucidate the function of Grp78 and may be used as therapeutic agents in their own right.

Antibodies may also be raised against specific epitopes of the proteins according to the invention. An antibody, or other compounds, "specifically binds" to a protein when it binds with high affinity to the protein for which it is specific but substantially does not bind, or binds with only low affinity to other proteins. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of an antibody are well known in the art. Such immunoassays typically involve the formation of complexes between the specific protein and its antibody and the measurement of complex formation.

Antibodies of the invention may be antibodies to human polypeptides or fragments thereof. For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which maintain their binding activity for a polypeptide encoded by a polynucleotide of the invention, a polypeptide of the invention or a fragment thereof. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies. Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanised antibodies.

Antibodies suitable for use as inhibitors of the invention can be produced by any suitable method. Means for preparing and characterising antibodies are well known in the art, see for example Harlow and Lane (1988) "Antibodies: A Laboratory Manual", cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. For example, an antibody may be produced by raising antibody in a host animal against the whole polypeptide or a fragment thereof, for example an antigenic epitope thereof, herein after the "immunogen".

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein (1975) *Nature* **256**, 495-497).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. Camels, llamas or sharks may be used if it is desired to isolate single chain antibodies. Thus, nanobodies may be used in the invention as inhibitors of Grp78. Nanobodies are derived from naturally occurring single-chain antibodies. The structure of such antibodies consists of a single variable domain (VHH), a hinge region, and two constant domains (CH2 and CH3).

If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Humanized antibodies may be obtained by replacing components of a non-human antibody with human components, without substantially interfering with the ability of the antibody to bind antigen.

Defined chemical entities, peptide and peptide mimetics, oligonucleotides or natural products may also be suitable inhibitors. A suitable inhibitor may be a chemical compound, for example a small molecule or an aptamer.

An inhibitor of Grp78 may act via an antisense mechanism or via an RNA interference mechanism (RNAi).

An inhibitor of Grp78 which acts via an antisense mechanism may comprise a polynucleotide which has substantial complementarity to all or part of an mRNA of such an enzyme. A polynucleotide which has substantial sequence complementarity to all or part of an mRNA of Grp78 is typically one which is capable of hybridizing to such an mRNA. If the inhibitor has substantial complementarity to a part of the mRNA

of Grp78, it generally has substantial complementarity to a contiguous set of nucleotides within that mRNA.

There are, generally speaking, two antisense approaches which may be used in the invention

In one approach, a vector is used which allows for the expression of a polynucleotide which has substantial sequence complementarity to all or part of an mRNA of Grp78 (i.e. a polynucleotide which can hybridize to an mRNA of such a gene). This results in the formation of an RNA-RNA duplex which may result in the direct inhibition of translation and/or the destabilization of the target message, by rendering it susceptibility to nucleases, for example. The vector will typically allow the expression of a polynucleotide which hybridizes to the ribosome binding region and/or the coding region of the mRNA of Grp78.

Alternatively, an oligonucleotide may be delivered which is capable of hybridizing to the mRNA of Grp78. Antisense oligonucleotides are postulated to inhibit target gene expression by interfering with one or more aspects of RNA metabolism, for example processing, translation or metabolic turnover. Chemically modified oligonucleotides may be used and may enhance resistance to nucleases and/or cell permeability.

In the first approach, the vector is capable of expressing a polynucleotide which has substantial sequence complementarity to all of part of an mRNA of Grp78. Such a polynucleotide will be capable of hybridizing to the mRNA. Typically, such a polynucleotide will be an RNA molecule. Such a polynucleotide may hybridize to all or part of the mRNA of Grp78. Generally, therefore the polynucleotide will be complementary to all of or part of the such an mRNA. For example, the polynucleotide may be the exact complement of such an mRNA. However, absolute complementarity is not required and preferred polynucleotides which have sufficient complementarity (i.e. substantial complementarity) to form a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to the mRNA of Grp 78 under conditions of medium to high stringency, such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

It is preferred that the polynucleotide hybridizes to a region of an mRNA of

Grp78 corresponding to a coding sequence. However, a polynucleotide may be employed which hybridises to all or part of the 5'- or 3'-untranslated region of such an mRNA. The polynucleotide will typically be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 600 or 700 nucleotides in length or even up to a few nucleotides, such as five or ten nucleotides, shorter than the full-length mRNA of Grp78.

The polynucleotide, (i.e. the "antisense" polynucleotide), may be expressed in a cell from a suitable vector. A suitable vector is typically a recombinant replicable vector comprising a sequence which, when transcribed, gives rise to the polynucleotide (typically an RNA). Typically, the sequence encoding the polynucleotide is operably linked to a control sequence which is capable of providing for the transcription of the sequence giving rise to the polynucleotide. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a sequence giving rise to an antisense RNA is ligated in such a way that transcription of the sequence is achieved under conditions compatible with the control sequences.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for transcription to occur and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of antisense RNA, or used to transfect or transform a host cell. The vector may also be adapted for used *in vivo*, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as b-actin promoters, may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art. Preferred promoters are tissue specific promoters, for example promoters driving expression specifically within vascular tissue.

Vectors may further include additional sequences, flanking the sequence giving rise to the antisense polynucleotide, which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination.

Examples of suitable viral vectors include retroviruses, including lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses. Gene transfer techniques using such viruses are will known to those skilled in the art. Retrovirus vectors, for example, may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

In the antisense oligonucleotide approach, a suitable oligonucleotide will typically have a sequence such that it will bind to an mRNA of Grp78. Therefore, it will typically have a sequence which has substantial complementarity to a part of such an mRNA. A suitable oligonucleotide will typically have substantial complementarity to a contiguous set out of nucleotides within the mRNA of Grp78. An antisense oligonucleotide will generally be from about 6 to about 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length.

Generally the oligonucleotide used will have a sequence that is absolutely complementary to the target sequence. However, absolute complementarity may not be required and in general any oligonucleotide having sufficient complementarity (i.e. substantial complementarity) to form a stable duplex (or triple helix as the case may be) with the target nucleic acid is considered to be suitable. The stability of a duplex (or triplex) will depend *inter alia* on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity between the antisense oligonucleotide and the target sequence.. The system can tolerate less complementarity when longer oligonucleotides are used. However oligonucleotides, especially oligonucleotides of from 6 to 40 nucleotides in length, which have sufficient complementarity to from a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

Antisense oligonucleotides may be chemically modified. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides.

An inhibitor suitable for use in the invention may act via an RNA interference (RNAi) mechanism. Such an inhibitor is typically a double-stranded RNA and has a sequence substantially similar to part of an mRNA of an mRNA for Grp78. Preferred inhibitors of this type are typically short, for example 15mers to 25mers, in particular 18mers to 22mers, preferably 21mers.

Generally the RNAi inhibitor will have a sequence that is absolutely complementary to the target sequence. However, absolute complementarity may not be required. In general an RNAi inhibitor which has a sequence that has sufficient complementarity (i.e. substantial complementarity) such that it would form a stable duplex with the target nucleic acid is considered to be suitable. The stability of such a duplex will depend *inter alia* on the sequence and length of the sequence of the RNAi inhibitor and the degree of complementarity between the RNAi inhibitor and the target sequence. RNAi inhibitors of from 15 to 25 nucleotides in length, which have a sequence which has sufficient complementarity to from a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly suitable for use in the present invention.

The RNAi inhibitor may have a sequence which hybridizes to the mRNA of Grp78 under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

The use of short inhibitors of this type is preferred because such inhibitors do not appear to trigger viral defence mechanisms of higher organisms. Such inhibitors can be used to inhibit translation of the mRNA..

Alternatively, small fragments of sequence encoding Grp78 (or a sequence substantially similar thereto) may be provided, cloned back to back in a suitable vector. The vectors described above are suitable for expression of such back to back sequences. Expression of the sequence leads to production of the desired double-stranded RNA.

Any suitable method may be carried out in order to determine whether a test substance is an inhibitor of a Grp78 (or a NOS). It will be apparent to those skilled in the art that there are many alternative ways of determining whether a test substance is an inhibitor of Grp78.

Generally, a method of the invention will be suitable for performing in a single reaction vessel, preferably in a single well of a plastics microtitre plate. Preferably, therefore, a method of the invention will be suitable for use as a high throughput screening technique.

A suitable technique for determining whether a substance is an inhibitor of Grp78 comprises:

(i) providing a polynucleotide construct, which construct comprises a Grp78 promoter, or functional variant thereof, operably linked to a coding sequence;

(ii) contacting the polynucleotide construct with the test substance under conditions which in the absence of the test substance would lead to expression of the coding sequence; and

(iii) determining whether the test substance inhibits expression of the coding sequence thereby to determine whether the test substance is an inhibitor of Grp78 expression.

A Grp78 promoter will be readily available to those skilled in the art. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a Grp78 promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the Grp78 promoter sequence. The term "Grp78 promoter", in this context, covers naturally occurring promoters and allelic variants and functional variants thereof. A functional variant of a Grp78 promoter is a sequence which shares sequence identity with a native Grp78 promoter (or allelic variant thereof), but which retains the ability to drive the expression of a coding sequence to which it is operably linked. The functional variant may be from a homolog or an ortholog.

The coding sequence may be any coding sequence, including that of the Grp78 gene itself. Typically, however, it may be more convenient to use the coding sequence of a so-called reporter gene, as those coding sequences encode readily identifiable polypeptides. For example, the coding sequence may encode a polypeptide which may be identified colorimetrically, for example β-glucoronidase (GUS), or fluorescently, for example a green fluorescent protein (GFP) or luciferase.

The construct is placed under conditions that in the absence of a test substance would lead to the expression of the coding sequence. It may be possible to carry this out using a suitable cell-free extract. The extract would be one which allows transcription and translation of the reporter polypeptide in the absence of the test substance. More usually, however, the method is carried out using a cell harbouring the Grp78 promoter:coding sequence construct.

The skilled person may thereby readily determined whether or not the test substance is capable of inhibiting a Grp78 and therefore whether or not it will be useful in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent and/or useful in the treatment of a cancer

A typical method is carried out as follows:

- a defined number of cells are inoculated, in for example 100µl of growth medium, into the wells of a plastics micro-titre plate in the presence of a substance to be tested;
- optical density (OD), for example at 590nm, may be measured, as may expression of the coding sequence, using a method appropriate for the particular coding sequence;
- the micro-titre plates are covered and incubated at 37°C for 3 days in the dark for an appropriate amount of time, for example, 24 or 48 hours;
- the OD is read again and expression of the coding sequence determined, allowing the effect of the test substance to be determined.

Control experiments can be carried out, in which the substance to be tested is omitted. In addition, the substance may be tested with any other known promoter to exclude the possibility that the test substance is a general inhibitor of gene expression.

The method of the invention may involve determining whether a test substance is capable of binding to a Grp78 (or to a functional variant thereof). Thus, the ability to bind to a Grp78 may be indicative of a substance which is an inhibitor of a Grp78. In other words, by identifying a substance which binds to a Grp78, it may thereby be possible to identify a substance which is an inhibitor of a Grp78.

The invention further provides a method for identifying an inhibitor of Grp78 activity comprising:

(i) providing a Grp78 or a functional variant thereof;

(ii) contacting the Grp78 with a test substance under conditions that in the absence of the test substance would permit activity of the Grp78; and

(iii) determining whether the test substance inhibits activity of the Grp78 thereby to determine whether the test substance is an inhibitor of Grp78 activity.

Any suitable format may be used for identifying an inhibitor of Grp78. Grp78 has ATPase activity and this property may be used to carry out a method of the invention.

Recombinant Grp78 can be provided and its ATPase activity determined both in the absence and presence of a test substance. Any Grp78 can be used or an active fragment or functional variant thereof. Preferably, any such fragment or variant will have ATPase activity.

Suitable Grp78 may be from a human or a non-human source. If the Grp78 is used from a non-human source, mammalian Grp78 sequences are preferred. The expression of recombinant Grp78 and the determination of its ATPase activity are described in King *et al*, Protein Expression and Purification 22, 148-158 (2001) and Carlino *et al.*, Proc. Natl. Acad. Sci. USA 89, 2081-2085 (1992). The nucleic acid and amino acid sequences for the human Grp78 are set out in GenBank accession number M19645 [gi:183644].

The assay may be carried out with a functional variant or active fragment of a Grp78. Suitable functional variants and active fragments will typically be substantially similar to a Grp78, for example the human Grp78. A polypeptide which is substantially similar to a Grp78 is one which shares sequence similarity with that gene product and also retains similar enzymatic activity thereto (for example with respect to ATPase activity). Similarly, a fragment suitable for use in the assay described above will also retain similar enzymatic activity to a Grp78 polypeptide (or a polypeptide substantially similar thereto) and preferably will exhibit ATPase activity.

Polypeptides substantially similar to a Grp78 may thus be used in the assays of the invention. Such polypeptides will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98 or at least 99% sequence identity with a Grp78 calculated over the full length of those sequences. However, the identity may be

calculated over a shorter length, for example 20, 50, 100 or more amino acids. If identity is calculated over a shorter length, it is typically done so over a contiguous length of amino acids. The determination of sequence identity is discussed below.

A functional variant may be a naturally occurring sequence, such as an allelic variant of a Grp78. An allelic variant will generally be of human or non-human mammal, for example bovine or porcine, origin.

Alternatively, a functional variant of a Grp78 may have a non-naturally occurring sequence. A non-naturally occurring polypeptide which is substantially similar to a Grp78 may be a modified version of one of those polypeptide, obtained by, for example, amino acid substitution, deletion or addition. Up to 1, up to 5, up to 10, up to 50 or up to 100 amino acid substitutions or deletions or additions, for example, may be made. Thus, a fragment of a Grp78 may be used in an assay of the invention.

Typically, if substitutions are made, the substitutions will be conservative substitutions, for example according to the Table set out below.

Typically, the activity of the Grp78 is initiated by addition of a suitable substrate, for example ATP. As a control the progress of the assay can be followed in the absence of the test substance.

Also the test substance may used in an assay with any other known enzyme to exclude the possibility that the test substance is a general inhibitor of enzyme activity.

In addition to the Grp78 and a suitable substrate, the reaction mixture can contain a suitable buffer, suitable cofactors and suitable divalent cations as cofactors. A suitable buffer includes any suitable biological buffer that can provide buffering capability at a pH conducive to the reaction requirements of the Grp78.

The assay of the invention may be carried out at any temperature at which Grp78, in the absence of any test substance or inhibitor, is active. Typically, however, the assay will be carried out in the range of from 25°C to 42°C, preferably at about 37°C.

Measures of enzymatic activity, for example ATPase activity, are generally known to those skilled in the art, including equilibrium constants, reaction velocities of the appearance of reaction products or the consumption of reaction substrates, reaction kinetics, thermodynamics of reaction, spectrophotometric analysis of reaction products, detection of labelled reaction components, etc. See, generally, Segel, Biochemical Calculations, 2nd Edition, John Wiley and Sons, New York (1976); Suelter, A Practical

Guide to Enzymology, John Wiley and Sons, New York (1985).

Preferred inhibitors identified in the assays described above are those which inhibit expression of a Grp78 polynucleotide and/or activity of a Grp78 polypeptide by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the inhibitor of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}.

The percentage inhibition represents the percentage increase or decrease in expression/activity in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition and concentration of inhibitor may be used to define an inhibitor of the invention, with inhibition at lower concentrations being preferred.

A further method for determining whether a test substance is an inhibitor of Grp78 comprises:

- providing a population of cells which is capable of generating NO;
- optionally subjecting the cell population to cellular stress;
- contacting the cell population with a test substance under conditions that, in the absence of the test substance, lead to the generation of NO by the cell population; and
- determining the proportion of the cell population which dies thereby to determine whether the test substance is an inhibitor of Grp78 and therefore whether it is capable of inhibiting NO/Grp78 mediated resistance to a chemotherapeutic agent or a radiotherapeutic agent.

The cell population may be subjected to any cellular stress. For example, the cells may be contacted with a chemotherapeutic agent or a toxin, subjected to radiation, for example UV irradiation, subjected to anoxia (or low oxygen conditions), subjected to temperature stress (low or high temperatures, for example temperatures lower or higher than from about 28°C to about 37°C) or subjected to low glucose concentrations. In particular, the cell population may be contacted with thapsigargin or an analog thereof. A number of chemotherapeutic and radiotherapeutic agents are mentioned below which may be used to induce cellular stress as required in the assay.

Generation of NO leads to the up-regulation of Grp78 which will protect the cell population from the cellular stress, for example thapsigargin toxicity. However, if the test substance is an inhibitor of Grp78, that protection will be decreased and a larger

proportion of the cell population will die as compared to the proportion of the cell population that dies in the absence of such a test substance. That is, the degree to which the cell population is sensitized to the cellular stress, for example thapsigargin toxicity, is determined. Control experiments carried out in the absence of the test substance will allow the degree of sensitization to be determined.

The assay is carried out under conditions that in the absence of the test substance, would lead to, or maintain, generation of NO by the cell population.

The population of NO-generating cells may be any suitable cell population that is capable of generating NO. Preferably, the cell population will be a population of cells in which NO production can be manipulated and controlled.

Two preferred types of cells are cells in which either ecdysone or tetracycline responsive systems have been engineered. Two suitable cell lines are the EcR293 clone 11 cell line) and the Tex 293 clone 22 cell line (Xu *et al.*, Nat. Cell. Biol. 2, 339-345, 2000; Xu *et al.*, FASEB J. 10, 1096/fj.01-0590fje, 2001; WO-A-01/09307 [PCT/GB00/02926]; WO-A-01/09342 [PCT/GB00/02924]; and WO-A-01/09375 [PCT/GB00/02932]) which are capable of regulatable high level NO generation.

Steroid hormones are small hydrophobic molecules that can diffuse through the plasma membrane of cells where they can bind reversibly to specific steroid-hormone-receptor proteins in the cytoplasm or nucleus. The binding of hormone activates the receptor, enabling it to bind with high affinity to specific DNA sequences that act as transcriptional enhancers. This binding increases the level of transcription from certain nearby genes.

A pulse of the insect steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing effects such as chromosomal puffing within minutes of hormone addition. Mediating this response is the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP).

Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of a cell with a functional ecdysone receptor (a heterodimer of EcR and USP) and an ecdysone responsive construct and treatment of the cell with ecdysone or an analog thereof.

A tetracycline responsive system can be created in cultured mammalian cells by cotransfection of a cell with a plasmid encoding a tetracycline repressor protein (tetR)

and a plasmid containing a tetracycline responsive element linked to a promoter. The promoter sequence is used to drive heterologous gene expression. The tetracycline responsive element comprises particular DNA sequences called tetracycline operator sites, which can bind a homodimer of tetR. If those sequences are positioned between a promoter and a coding sequence in a construct, the presence of tetR bound to a tetracycline operator site will prevent the promoter driving expression of the coding sequence. However, when tetracycline is added to cells the tetracycline binds to tetR homodimers leading to a conformational change in tetR, such that it is unable to bind a tetracycline operator site. The tetR:tetracycline complex dissociates from the Tet operator site and allows the promoter to drive expression of the coding sequence.

Polynucleotide constructs may be constructed which are responsive to ecdysone or an analog thereof or which are responsive to tetracycline or an analog thereof.

Ecdysone responsive constructs comprise a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence codes for a NOS or a functional variant or fragment thereof.

Tetracycline responsive constructs comprise a promoter operably linked to one or more teracycline operator site sequences and a coding sequence in that order, wherein the coding sequence codes for a NOS or a functional variant or fragment thereof.

The constructs may comprise DNA or RNA. They may also include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the invention, it is to be understood that the constructs described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of constructs of the invention. Constructs of the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved

under conditions compatible with the regulatory sequence.

A promoter for use in an ecdysone-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the steroid hormone ecdysone or an analog thereof.

The promoter may be a naturally occurring promoter from a *Drosophila melanogaster* or other insect ecdysone-responsive gene. Alternatively, the promoter may be a non-naturally occuring promoter. A non-naturally occuring promoter may be used which comprises a minimal promoter and an ecdysone-responsive element (EcRE). An EcRE is a nucleotide sequence to which a functional ecdysone receptor can bind in the presence of ecdysone. Suitable minimal promoters include the minimal heat shock promoter.

An ecdysone-responsive promoter may comprise more than one EcRE, for example 2 to10 elements or more preferably 4 to 6 elements. The sequence of an EcRE will depend on the exact functional ecdysone receptor used. If a modified functional ecdysone receptor is used (see below) it may be appropriate to use a modified EcRE (see No *et al.*, *Proc. Natl. Acad. Sci. USA*, **93**: 3346-3351). The EcRE(s) and minimal promoter sequences do not have to be immediately adjacent. Because EcREs function as transcriptional enhancers, they can be placed some distance upstream, for example from 1, 10 or 25 nucleotides to 30, 40, 50, 100, 500 or 1000kb upstream of a minimal promoter. EcREs could even be placed further than 1kb upstream of a minimal promoter. Generally, if multiple copes of an EcRE are used, the mutiple copies will be arranged in an array, one after the other.

Constructs of the invention may be responsive to ecdysone [(*2β, 3β, 5β, 22R*)-2,3,14,22,25-pentahydroxycholest-7-en-6-one] or an analog thereof. Suitable analogs of ecdysone for use in the invention include muristerone A [2β, 3β ,5α, 11α, 14, 20, 22-heptahydroxy-5β, 7-cholesten-6-one] or ponasterone A [(*2β, 3β, 5β, 22R*)-2, 3, 14, 20, 22, 25-pentahydroxycholest-7-en-6-one] and GS™-E (Invitrogen, San Diego, CA; see also Dhadialla *et al.*, 1998, Ann. Rev. Entomol. **43**: 545-569).

A promoter for use in an tetracycline-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the antibiotic tetracycline or an analog thereof.

Generally, the choice of promoter will depend on the host cell to be used for expression of the coding sequence. Typically, expression in mammalian cells, for

example human cells will be required and thus a mammlian promoter will be preferred. Mammalian promoters, such as β-actin promoters, may be used. Tissue-specific promoters may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art. Constitutive promoters, for example the CMV promoter, are preferred.

Tetracycline-responsive constructs comprise one or more tetracycline operator site ($TetO_2$) sequences, situated between the promoter and coding sequence. For example, two, three, four or even up to ten $TetO_2$ sequences may be used. Typically, if more than one $TetO_2$ site is used those sites will be arranged in the form of an array. However, other intervening nucleotide sequences may be situated between individual $TetO_2$ sites. For example one, two, three, four, five, up to ten or up to 15 nucleotides may intervene between any two $TetO_2$ sites.

The $TetO_2$ sequence is 5'-TCCCTATCAGTGATAGAGA-3' (Hillen and Berens, 1994, Annu. Rev. Microbiol. 48, 345-369; Hillen *et al.*, 1983, J. Mol. Biol. 169, 707-721) or a functional variant thereof. The $TetO_2$ sequence or a functional variant thereof is capable of being bound by a homodimer of tetR or a functional variant thereof.

A functional variant of the $TetO_2$ sequence is a sequence which is similar to that of the $TetO_2$ sequence and which remains capable of binding a homodimer of tetR or a functional variant thereof. The affinity of tetR for the $TetO_2$ sequence is $K_B = 2x10^{11}$ M^{-1} (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994, *supra*). The binding affinity of tetR for a functional variant of the $TetO_2$ sequence may be substantially the same as that of tetR for the $TetO_2$ sequence. Alternatively, tetR may have a binding affinity for a functional variant of the $TetO_2$ sequence which is greater or less than that of tetR for the $TetO_2$ sequence. For example, the affinity of tetR for a functional variant of the $TetO_2$ sequence may be from $K_B = 2x10^9$ M^{-1} to $2x10^{13}$ M^{-1} or more preferably from $2x10^{11}$ M^{-1} to $2x10^{12}$ M^{-1}.

A functional variant of $TetO_2$ typically comprises a sequence substantially similar to that of the $TetO_2$ sequence. Thus, a functional variant of $TetO_2$ will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or

at least 99% sequence identity to the $TetO_2$ sequence, calculated over the full length of those sequences.

A functional variant of the $TetO_2$ sequence may be a modified version of that sequence obtained by, for example, nucleotide substitution or deletion. Up to 1, up to 2, up to 3, up to 4, up to 5, up to 6 or more nucleotide substitutions or deletions or combinations thereof may be made to the $TetO_2$ sequence to produce a functional variant of that sequence.

Constructs of the invention may be responsive to the antibiotic tetracycline or an analog thereof. Tetracycline binds to tetR homodimers, such that the tetR:tetracylcine complex dissociates from the $TetO_2$ sequence. The association constant of tetracycline to tetR is $3 \times 10^9\ M^{-1}$. Preferred analogs of tetracycline will have an association constant substantially similar to or greater than that of tetracycline for tetR. Suitable analogs of tetracycline include doxycycline. Doxycycline exhibits similar dose response and induction characteristics with constructs of the invention, but has a longer half-life than tetracycline (48 hours vs. 24 hours respectively).

The coding sequence used in both ecdysone- and tetracycline-responsive contructs of the invention can be any sequence which encodes a NOS or a functional variant thereof. The phrase "nitric oxide synthase" is intended to include all naturally occuring forms of iNOS, nNOS and eNOS as well as variants which retain NOS activity, for example variants produced by mutagenesis techniques. Preferably the coding sequence encodes a NOS of mammlian origin for example rodent (including rat and mouse) or human. Most preferably the coding sequence encode the human iNOS (GenBank accession number: X73029, Coding sequence 226-3687), human nNOS (GenBank accession number: U17327, Coding sequence 686-4990) or human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) or a functional variant of any one of those enzymes.

A functional variant of a NOS is any polypeptide which demonstrates NOS activity, for example a fragment of a NOS. A coding sequence which codes for a functional variant of a NOS may be, for example a fragment of a full length NOS coding sequence. A fragment may be of any length, so long as the polypeptide for which it codes has NOS activity.

A functional variant of a NOS typically comprises a sequence substantially similar to that of the naturally occurring form of the relevant NOS sequence. Thus, a

functional variant of a NOS will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the relevant NOS sequence, calculated over the full length of those sequences.

Thus, the coding sequence may be modified by nucleotide substitutions or deletions. For example up to 1, 2 or 3 to 10, 25, 50, 75 or 100 substitutions or deletions or combinations thereof may be made to produce a functional variant of a NOS. A polynucleotide encoding a NOS may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes for a polypeptide which has NOS activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as shown in the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Sequence identity may be calculated as follows. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Both types of construct can be incorporated into a recombinant replicable

vector. The vector may be used to replicate the construct in a compatible host cell. A vector may also provide for expression of the NOS coding sequence when the vector is harboured by an appropriate host cell. The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication and optionally a regulator of the ecdysone-responsive promoter or promoter used in a tetracycline-responsive construct.

The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene for selection in bacterial cells or a G418 or a zeocin resistance gene for selection in mammlian cells.

Vectors which incorporate an ecdysone-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be induced in the presence of ecdysone or an analog thereof, a cell harbouring an ecdysone inducible construct must preferably also be capable of expressing a functional ecdysone receptor.

As described above, the wild type *Drosophila* functional ecdysone receptor is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP). Thus cells of the invention may be capable of expressing EcR and USP. However, replacement of EcR's natural heterodimeric partner USP with its mammlian homologue retinoid X receptor gives a heterodimer which can give more potent induction of an ecdysone responsive promoter. Thus cells of the invention may be capable of expressing EcR and RXR. It will be clear that cells of the invention may be capable of expressing functional variants of either subunit of the heterodimer. Functional variants of EcR and USP/RXR are polypeptides which can heterodimerise with their partner and can, when heterodimerised, allow ecdysone-responsive dimerisation to occur. In some cases functional variants may bind to non-wild type EcREs. Examples of functional variants and modified EcREs are described in No *et al. Proc. Natl. Acad. Sci. USA*, 93: 3346-3351.

Preferred cells are human cells. Particularly preferred cells are EcR293 cells (Invitrogen, San Diego, CA; Catalogue No: R650-07; EcR293 is a derivative of the human fetal kidney cell line HEK293 (ECACC accession number 85/20602)). EcR293

cells are particularly suitable as they stably transformed with the vector pVgRXR. That vector is capable of expressing a functional variant of EcR, VgEcR, and RXR in mammalian cells and thus allows the expression of a functional ecdysone receptor. Other suitable cell lines include EcR-CHO and EcR-3T3 (Invitrogen, San Diego, CA; Cat. Nos: R660-07 and R680-07 respectively). Those two cell lines are stably transformed with the same vector, pVgRXR, as the EcR293 cell line.

Vectors which incorporate an tetracycline-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence in the presence of tetracycline or an analog thereof. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be regulated such that expression does not occur in the absence of tetracycline, a cell harbouring a tetracycline-inducible construct must preferably also be capable of expressing the tetracycline repressor protein (tetR) or a functional variant thereof.

A functional variant of tetR is a polypeptide which is similar to tetR and which remains capable of binding, as a homodimer, the $TetO_2$ site or a functional variant thereof and tetracycline or an analog thereof. Typically, the binding affinity of a functional variant sequence of tetR for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof may be substantially the same as the binding affinity of the tetR polypeptide for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof. Alternatively, a functional variant sequence may have a binding affinity which may be greater or less than that of the tetR polypetide.

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa (Hillen and Berens, 1994, *supra*). A functional variant of tetR typically comprises an amino acid sequence substantially similar to that of the tetR sequence. Thus, a functional variant of a tetR will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to tetR, calculated over the full length of those sequences. The calculation of sequence identities is described above.

A functional variant of the tetR sequence may be a modified version of that sequence obtained by, for example, amino acid substitution or deletion. Up to 1, up to

10, up to 20, up to 50, up to 75, up to 100 or more amino acid substitutions or deletions or combinations thereof may be made to the tetR sequence to produce a functional variant of that sequence. Substitutions are preferably made which result in a conservative amino acid substitution, for example as shown in the Table above.

Preferred cells are human cells. Particularly preferred cells are T-REx cells (Invitrogen, San Diego, CA; Catalogue Nos: R710-07, R712-07, R714-07 and R716-07). T-Rex cells are particularly suitable as they stably transformed with the plasmid pcDNA6/TR which generates high level expression of the tetR polypeptide. However, any cell line can be used which expresses tetR or a functional variant thereof.

Using the cells described above, the method of the invention may be carried out by, for example:

- growing the cells in the presence of a suitable inducer, muristerone A or tetacycline;
- simultaneously subjecting the cells to cellular stress (for example contacting them with thapsigargin or an analog thereof) and contacting them with a test substance;
- determining the proportion of the cell population that is killed.

The proportion of cell viability may be determined by any convenient technique. For example, a simple colourimetric assay of LDH may be appropriate. Suitable control assays may be carried out. For example, the assay can be carried out in the absence of a test substance to determine a basal level of cell death.

Use of this technique will permit the identification of substances that protect against resistance to the toxicity of a chemotherapeutic agent or a radiotherapeutic agent. Further assays, for example those set out above may be used to further determine whether the substance identified is an inhibitor of Grp78. Alternatively, assays may be carried out in order to determine whether the substance identified is an inhibitor of a NOS. Suitable assays for determining whether a substance is an inhibitor of a NOS are well known to those skilled in the art.

All of the assays set out above may be carried out by replacing thapsigargin with any other substance or conditions that induces cellular stress. In particular such substances may, like thapsigargin, inhibit the Ca^{2+}-sequestering ATPase of the endoplasmic reticulum. The Ca^{2+} ionophore A23187 may be used instead of thapsigargin in any of the assays set out above. Alternatively, if the assay is a cell-

based assay, cells used in the assay may be subjected to stress by manipulation of the conditions used to carry out the assay. For example, cells may be subjected to glucose starvation or oxygen deprivation. Such conditions may be used instead of the addition of thapsigargin or some other stress-inducing substance.

For all of the assays described above, control experiments can be carried out, for example in which the test substance is omitted. Also, substances so-identified may be tested with other known promoters (to exclude the possibility that the test substance is a general inhibitor of gene expression) and other known polypeptides (to exclude the possibility that the test substance is a general inhibitor of enzyme activity, for example a protease).

Any suitable format may be used for carrying out the assays. The reaction mixtures may contain a suitable buffer. A suitable buffer includes any suitable biological buffer that can provide buffering capability at a pH conducive to the reaction requirements of the enzyme in question. Typically, the assays may be adapted so that they can be carried out in a single reaction vessel and more preferably can be carried out in a single well of a plastics microtitre plate and thus can be adapted for high through-put screening.

Suitable candidate substances for screening for inhibitors of Grp78 include combinatorial libraries, defined chemical identities, peptide and peptide mimetics, oligonucleotides and natural product libraries. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition tested individually.

Furthermore, antibody products, for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies) which are specific for differentially expressed polypeptides may be used. Antibody fragments may be used, including Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (such as those described above). Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanized antibodies.

In addition, suitable test substances may include the products so-called phage-display libraries, where recombinant phage are used to express single-chain fragments (scFv) of diverse antibody complexity. Such libraries can be panned against a Grp78 (or an active fragment or a functional variant thereof) in a method of the invention,

thereby to identify an inhibitor of a Grp78.

Similarly, a Grp78 (or active fragment or functional variant thereof) may be used as a substrate to pan for aptamers that bind to such polypeptides/fragments. Typically such aptamers will bind specifically to such the Grp78. Aptamers suitable for use in the invention may be identified in a Systematic Expansion of Ligands by EXponential enrichment (SELEX) technique. Such apatamers may be candidate inhibitors of a Grp78.

Preferred substances identified in the above assays are those which lead to a change in activity in the assay (as measured by for example cell death, reporter gene expression or Grp78 activity) of at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% (if a decrease in activity is indicative of the presence of an inhibitor of Grp78) or of at least 10%, at least 25%, at least 50%, at least 100%, at least 200%, 500% or at least 1000% (if an increase in activity is indicative of the presence of an inhibitor of Grp78) at a concentration of the substance of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1},500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}.

The percentage change represents the percentage change in activity in the assay, as measured in whatever way is required by the assay, in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage change of activity and concentration of inhibitor may be used to define an inhibitor of the invention (i.e. an inhibitor of Grp78), with change of activity (inhibition) at lower concentrations being preferred.

Use of the methods of the invention for identifying an inhibitor of Grp78 described above has led to the identification of an scFv which is an inhibitor of Grp78. This scFv is encoded by the nucleic acid sequence set out in SEQ ID NO: 1. The amino acid sequence of the scFv is set out in SEQ ID NO: 2. These sequences are also set out in Figure 7. Accordingly, the invention thus an scFv having the amino acid sequence of SEQ ID NO: 2 or Figure 7 or a functional variant of either thereof. This scFv is an inhibitor of Grp78.

It has been shown that this scFv can attenuate the Grp78-mediated protection against thapsigargin, suggesting that the scFv may be used in the treatment of a cancer and/or in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent. That is to say, the scFv having the amino acid sequence set out

in SEQ ID NO: 2 may be used as a Grp78 inhibitor in such treatment as is described below in relation to Grp78 inhibitors in general.

The present invention also provides a functional variant of the scFv having the amino acid sequence of SEQ ID NO: 2. A functional variant of the scFv will typically be substantially similar to the scFv. A polypeptide which is substantially similar to the scFv is one which shares sequence similarity with that the scFv and also retains similar activity thereto, i.e. anti-Grp78 activity. That is to say, a functional variant of the scFv of the invention will be capable of binding Grp78 (or a functional variant of a Grp78 as defined above). A functional variant may have greater scFv or less scFv anti-Grp78 activity than the scFv having the sequence of SEQ ID NOs:1 and 2 (Figure 7). For example, a functional variant may have a higher affinity for Grp78 (or a functional variant thereof), for example, 2 times, five times, ten times, or more than ten times the affinity for Grp78 (or a functional variant thereof) than the scFv of the invention.

Also, a functional variant of the scFv of the invention will typically also decrease viability and/or increase apoptosis of a Grp78-expressing cell. Such a decrease or increase refers to a decrease/increase in viability/apoptosis as compared with the viability/apoptotic state of a Grp78-expressing cell in the absence of the functional variant.

Polypeptides substantially similar to the scFv of the invention will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98 or at least 99% sequence identity with that sequence, calculated over its full length. However, the identity may be calculated over a shorter length, for example 20, 50, 100, 150, 200 or more amino acids. If identity is calculated over a shorter length, it is typically done so over a contiguous length of amino acids. The calculation of sequence identity is described above.

A polypeptide which is substantially similar to the scFv of the invention may have a non-naturally occurring sequence. A non-naturally occurring polypeptide which is substantially similar to the scFv of the invention may be a modified version of one of that polypeptide, obtained by, for example, amino acid substitution, deletion or addition. Up to 1, up to 5, up to 10, up to 50 or up to 100 amino acid substitutions or deletions or additions, for example, may be made. Thus, a functional variant of the scFv of the invention may be a fragment of that scFv.

Typically, if substitutions are made, the substitutions will be conservative

substitutions, for example according to the Table set out above. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. Deletions are preferably deletions of amino acids from one or both ends of the scFv of the invention.

The scFv of the invention and functional variants thereof may used useful in prophylaxis and therapy as set out below.

Polypeptides for use in the assays described above or for use as the scFv of the invention and functional variants and fragments thereor can be obtained, for example, recombinantly by any method known to those skilled in the art. Alternatively, polypeptides may be chemically synthesized. Synthetic techniques, such as a solid-phase Merrifield-type synthesis, may be preferred for reasons of purity, antigenic specificity, freedom from unwanted side products and ease of production. Suitable techniques for solid-phase peptide synthesis are well known to those skilled in the art (see for example, Merrifield et al., 1969, Adv. Enzymol 32, 221-96 and Fields et al., 1990, Int. J. Peptide Protein Res, 35, 161-214). In general, solid-phase synthesis methods comprise the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain.

Polypeptides (or fragments or variants thereof) suitable for use in the invention may be fused to a carrier polypeptide. Thus, additional amino acid residues may be provided at, for example, one or both termini of a Grp78 polypeptide, an scFv having the sequence of SEQ ID NO: 2/Figure 1, or a functional variant of either thereof for the purpose of providing a carrier polypeptide, by which the polypeptide can be, for example, affixed to a label, solid matrix or carrier. Thus a polypeptide for use in a method of the invention may be in the form of a fusion polypeptide which comprises heterologous sequences. Indeed, in practice it may often be convenient to use fusion polypeptides. This is because fusion polypeptides may be easily and cheaply produced in recombinant cell lines, for example recombinant bacterial or insect cell lines. In addition, fusion polypeptides may be easy to identify and isolate. Typically, fusion polypeptides will comprise a polypeptide sequence as described above and a carrier or linker sequence. The carrier or linker sequence derived from a human or non-human, source, for example a non-human source such as a bacterial source.

Polypeptides may be modified by, for example, addition of histidine residues, a T7 tag or glutathione S-transferase, to assist in their isolation. Alternatively, the carrier

polypeptide may, for example, promote secretion of the polypeptide from a cell or target expression of the polypeptide to the cell membrane. Amino acids carriers can be from 1 to 400 amino acids in length or more typically from 5 to 200 residues in length. The polypeptide may be linked to a carrier polypeptide directly or via an intervening linker sequence. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid or aspartic acid.

Suitable polypeptides for use in the methods of the invention may be chemically modified, for example, post translationally modified. For example they may be glycosylated or comprise modified amino acid residues. Polypeptides can be in a variety of forms of polypeptide derivatives, including amides and conjugates with polypeptides i.e. Grp78, the scFv of the invention or polypeptides substantially similar to either thereof may be so-modified.

Chemically modified polypeptides also include those having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized side groups include those which have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups and formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine.

Substances identified by a method of the invention (such as the scFv having the sequence of SEQ ID NO: 2/Figure 7), inhibitors of a NOS or inhibitors of Grp78 may be useful in prophylaxis or therapy. Thus, the invention provides a substance identified in a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 for use in a method of treatment of the human or animal body by therapy. In particular those substances may be used in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent and in the treatment of a cancer.

The invention also provides use of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent and in the treatment of a cancer.

The invention also provides a method of treating or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host or for treating a cancer,

which method comprises the step of administering to the host an effective amount of an inhibitor of Grp78 or an inhibitor of a NOS. The host may be a human or an animal.

The condition of a patient suffering from a cancer can be improved by administration of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78. A therapeutically effective amount of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 may be given to a patient in need thereof.

A substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 may be used in combination with a chemotherapeutic agent and/or a radiotherapeutic agent. Thus, the invention provides products containing a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 as a combined preparation for simultaneous, separate or sequential use in inhibiting or preventing resistance to a chemotherapeutic agent and/or a radiotherapeutic agent, in particular in the treatment of a cancer. The invention also provides use of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78 in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent, in particular in the treatment of a cancer. The invention also provides a method of treating or preventing a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of:

(i) an inhibitor of a NOS, an inhibitor of Grp78 or a substance identified by a method of the invention; and

(ii) a chemotherapeutic agent or a radiotherapeutic agent.

The host may be a human or an animal.

The condition of a patient suffering from a cancer can be improved by administration of products of the invention. A therapeutically effective amount of products of the invention may be given to a patient in need thereof.

In the invention, a chemotherapeutic agent and a radiotherapeutic agent may be used in combination with a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78.

A substance identified by a method of the invention, an inhibitor of a NOS, an inhibitor of Grp78 or products of the invention may be used in the treatment of any

cancer. Examples of cancers that may be treated according to the invention include primary and secondary cancers. The cancer may be, for example, a leukaemia, a lymphoma, a sarcoma, a carcinoma or an adenocarcinomas. Specific types of cancers that may be treated according to the invention include breast, colon, brain, lung, ovary, pancreatic, stomach, skin, testicular and tongue cancers.

Any pharmaceutically acceptable inhibitor of NOS can be used in the present invention. Typically, an inhibitor of a NOS enzyme is used. Competitive, non-competitive, reversible and irreversible inhibitors are suitable. The inhibitor may inhibit iNOS, eNOS and/or nNOS. Preferably, the inhibitor will selectively inhibit the NOS isoform expressed in the tumor to be treated.

Suitable inhibitors include L-arginine analogues, thiocitrullines, indazole derivatives, imidazole derivatives, hydrazine derivatives, thioureas, thiazoles, biotin derivatives and phenyl-substituted thiopene amidines.

Examples of suitable L-arginine analogues include methyl-L-arginine, N^G-nitro-L-arginine methyl esther (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), N^G-amino-L-arginine (L-NAA), N^w,N^w-dimethyl-L-arginine (ADMA), N^w,N^{w2}-dimethyl-L-arginine (SDMA), N^w-ethyl-L-arginine (L-NEA), N^w-methyl-L-homoarginine (L-NMHA), N^w-nitro-L-arginine (L-NOARG), N^δ-iminoethyl-L-ornithine (L-NIO), N^δ-iminoethyl-L-lysine (L-homo-NIO) and L-canavanine (L-CAN).

Examples of suitable thiocitrullines include S-methyl-L-thiocitrulline (SMTC), L-thiocitrulline (L-TC) and L-S-ethyl-thiocitrulline (Et-TC).

Examples of suitable indazole derivatives include indazole and 7-substituted indazoles such as 7-nitroindazole and 3-bromo-7-nitroindazole.

Examples of suitable hydrazine derivatives include aminoguanidine.

Examples of suitable imidazole derivatives include phenyl substituted imidazoles such as 1-phenyl-imidazole.

Examples of suitable thioureas include S-methylisothiourea sulphate, δ-(S-methylisothioureido)-L-norvaline (L-MIN), S-ethylisothiourea (SETU) and S-isopropylisothiourea (SIPT).

Examples of suitable thiazoles include 2-amino-thiazole and 2-amino-4,5-dimethyl thiazole.

Examples of suitable biotin derivatives include 2-iminobiotin.

The above NOS inhibitors are commercially available, or may be made by

analogy with known methods.

The inhibitor may be a pharmaceutically acceptable salt of one the above compounds. Suitable salts include salts with pharmaceutically acceptable acids, both inorganic acids such as hydrochloric, sulphuric, phosphoric, diphosphoric, hydrobromic or nitric acid and organic acids such as citric, fumaric, maleic, malic, ascorbic, succininc, tartaric, benzoic, acetic, methanesulphonic, ethanesulphonic, benzenesulphonic or p-toluenesulphonic acid. Salts may also be formed with pharmaceutically acceptable bases such as alkali metal (eg sodium or potassium) and alkali earth metal (eg calcium or magnesium) hydroxides and organic bases such as alkyl amines, aralkyl amines or heterocyclic amines.

Any suitable chemotherapeutic agent may be used in the invention. The term chemotherapeutic agent includes any substance which can be used to destroy cancer cells, for example by inhibiting/preventing such cells from growing or multiplying. Chemotherapeutic agents suitable for use in the invention include antimetabolites, agents that react with DNA, inhibitors of transcription enzymes, topoisomerase inhibitors, DNA minor-groove binding compounds, antimotic agents, bleomycin group antitumor antibiotics, antihormones, paclitaxels and photochemically activated agents.

Suitable chemotherapeutic agents may be DNA damaging agents, for example substances which are DNA alkylating and/or cross-linking agents or substances which are DNA binding/cleaving agents.

Suitable chemotherapeutic agents which are DNA alkylating and/or cross-linking agents include nitrosoureas, nitrogen mustards, mitomycins and platinum coordination compounds. Such substances typically have the ability to react covalently with DNA bases and to form inter- and intrastrand DNA cross-links. These compounds may also be responsible for the alkylation of proteins and protein-DNA linkages. The resulting lesions produced in the DNA result in the disruption of cell growth and function, ultimately leading to cell death.

Suitable nitrosoureas include carmustine USP (BiCNU), lomustine USP (CeeNU), tauromustine and streptozocin USP (Zanosar).

Suitable nitrogen mustards include cyclophosphamide USP (Cytoxan), ifosphamide (Ifex), mesna USP (Mesnex), mechlorethamine hydrochloride USP (Mustargen), chlorambucil USP (Leukeran), melphalan USP (Alkeran) and thiotepa USP (Thiotepa).

Suitable mitomycins include mitomycin C USP (Mutamycin), BMY-25067 and KW2149.

Suitable platinum coordination compounds include cisplatin USP (Platinol) and carboplatin USP (Paraplatin).

Substances which are DNA binding/cleaving agents (DNA interactive agents) may bind to DNA either as intercalators or as minor groove binders, hence inhibiting DNA dependent RNA synthesis. Such substances may also cleave DNA by forming free radicals in the immediate vicinity of the sugar-phosphate backbone. Activity as antitumor agents is typically related to the ability to induce irreparable lesions in DNA. For example, one suitable substance bleomycin generates oxygen free-radical species, whereas another suitable substance , esperamicin A_1, generates aryl diradical species, which abstract hydrogen atoms directly from the deoxyribose backbone.

Suitable DNA interactive substances include danorubicin hydrochloride USP (Cerubidine), doxorubicin USP (Adriamucin), idarubicin hydrochloride (Idamycin), mitoxanthrone hydrochloride USP (Novantrone), bleomycin sulfate USP (Blenoxane), epseramicin A_1, Adozelesin (U73, 975), dactinomycin USP (Cosmegen), plicamycin USP (Mithracin), and procarbazine hydrochloride USP (Matulane).

It will be apparent to those skilled in the art that analogs of the above mentioned DNA damaging agents may also be suitable for use in the invention as chemotherapeutic agents.

Any suitable radiotherapeutic agent may be used in the invention. A suitable radiotherapeutic agent will typically be a type of ionizing radiation, for example photon therapy, using X-rays or gamma rays for instance. X-rays may be produced by a linear accelerator or a betatron, for example. External beam radiotherapy may be used to focus the radiation on the cancer site. Gamma rays may be produced by the spontaneous decay of, for example, radium.

In addition, a radiotherapeutic agent may be applied externally during surgery. Further, a radiotherapeutic agent may be particle beam radiation (high energy transfer radiation), for example neutrons, pions or heavy ions. This involves the use of fast-moving subatomic particles, rather than photons.

A radiotherapeutic agent may be applied externally, i.e. b y external beam radiotherapy. Alternatively, a radiotherapeutic agent may be delivered to cancer cells my placing radioactive implants directly in a tumor or a body cavity. This is called

internal radiotherapy and includes brachytherapy, interstitial irradiation and intracavitary irradiation. In addition, radiolabelled antibodies may be used to deliver doses of radiation directly to the cancer site, i.e. radioimmunotherapy may be used.

A substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78, optionally in combination with a chemotherapeutic agent, are typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. This also applies to radiotherapeutic agents formulated for use in internal radiotherapy or radioimmunotherapy. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginte, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78, optionally in combination

with a chemotherapeutic agent or a radiotherapeutic agent, is administered to a patient. The dose of a substance identified by a method of the invention, an inhibitor of a NOS or an inhibitor of Grp78, optionally in combination with a chemotherapeutic agent or a radiotherapeutic agent, may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

The invention also provides a method for determining the presence of a cancer in a subject. Such a method comprises typing the gene encoding Grp78 within the subject. Typically, such typing will involve determining the level of expression of the Grp78 gene in the subject. Such expression typing may be carried out at the nucleotide, for example RNA level, such as by RNA blotting or reverse transcriptase-PCT, and/or at the polypeptide level, for example by use of an antibody.

Such a method of the invention may be carried out *in vivo*, although typically it is more convenient to carry it out *in vitro* on a sample derived from the subject. The sample typically comprises a body fluid of the individual and may for example be obtained using a swab, such as a mouth swab. The sample may be a blood, urine, saliva, cheek cell or hair root sample. Alternatively, the sample may be a tissue sample, for example from a biopsy.

Any such sample is typically processed before the method is carried out, for example nucleic acid extraction, such as extraction of genomic DNA or RNA, in particular mRNA may be carried out. RNA, such as mRNA, extracted from a sample may subsequently be converted into cDNA. The nucleic acid or protein in the sample may be cleaved either physically or chemically (e.g. using a suitable enzyme, such as a restriction endonuclease). The polynucleotide in the sample may be copied (or amplified), e.g. by cloning or using a PCR based method.

In such a method of the invention, increased expression of the Grp78 gene in the subject, as compared to the amount of expression of that gene in a subject which does not have a cancer, or as compared to non-cancerous tissue from the subject, indicates

the presence of a cancer in the subject. Thus, a biopsy may include tumor tissue and (non-cancerous) tissue surrounding the tumor tissue. Increased expression of Grp78 in the tumor tissue as compared to the level of Grp78 expression in the surrounding tissue may indicate that the tumor is cancerous.

An antibody, in particular the scFv of the invention or a functional variant thereof, may be used in such a method of the invention. In particular, tissue samples, for example a biopsy such as a punch biopsy, from a subject may be subjected to western blot analysis of immuncytochemistry using an antibody (such as the scFv or functional variant).

In such a method it may be possible to identify any kind of cancer, for example those forms of cancer mentioned above in relation to therapy.

The following Example illustrates the invention:

EXAMPLE

Materials and methods

Unless indicated otherwise, the methods used are standard biochemical techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, a Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Cloning

The NO inducible cell lines, EcR293 clone 11 subclone-1 and Tex293 clone 22 were prepared as described previously (Xu *et al.*, Nat. Cell. Biol. 2, 339-345, 2000; Xu *et al.*, FASEB J. 10, 1096/fj.01-0590fje, 2001; WO-A-01/09307 [PCT/GB00/02926]; WO-A-01/09342 [PCT/GB00/02924]; and WO-A-01/09375 [PCT/GB00/02932]). The Creator Cloning system (Clontech) was used to clone a full-length human Grp78 cDNA (Image clone 2286788) into two expression vectors: pLP-IRE2-EGFP-Grp78 (containing a CMV promoter and a neomycin selection marker) and pLP-TRE2-Grp78 (containing a tetracycline-inducible promoter). The latter construct was co-transfected with the hygromycin-selection plasmid pTK-Hyg (Clontech) into the MCF7 Tet-on cell line (Clontech) with (50 μg/ml. Hygromycin,Gibco) for the establishment of a

permanent cell line. Western blotting was used to detect Grp78 expression in cell lines using monoclonal anti-Grp78 antibody (Transduction Laboratories).

Assay of gene expression

cDNA microarray analysis was carried out by Incyte Genomics, Palo Alto, California. Grp78 regulation by NO was further supported by northern and western blotting using the methods described previously (Xu *et al.*, 2000, *supra*).

Promoter analysis

The 5-'promoter region of the human Grp78 gene was cloned into a luciferase reporter vector pGL3-basic (Promega) using PCR primers corresponding to the genomic sequence. The forward primer 5'-CCCGGGGTCACTCCTGCTGGTACCTAC-3' and reverse primer 5'-TCCTTCTTGTCCTCCTCCTAAGCTTCGCG-3' incorporated *Kpn*I (forward) and *Hind*III (reverse) sites to facilitate cloning. The detailed transfection and luciferase assay conditions were as described previously (Xu *et al.*, 2000, *supra*).

Thapsigargin (Tg) cytotoxicity experiments

Tg (Sigma) stock was dissolved in DMSO and diluted to the desired concentration prior to use. Cell viability was assayed by the LDH cytotoxicity detection kit (Roche). Cells were treated with 1% triton X-100 overnight to generate 100% killing, while untreated cells were used as negative controls. Grp78 antisense oligonucleotide administration (200 μM) was carried out at the same time as Tg treatment for breast cancer cell lines. The antisense phosphorothioate oligodeoxynucleotide:

5'- TGATCTCCACGCGGCCGTTCTTGAACACGCCG-3'

and the sense oligonucleotide:

5'-CGGCGTGTTCAAGAACGGCCGCGTGGAGATCA-3'

were designed from the human Grp78 sequence (Oswel).

NO induction in breast cancer cell line and nitrite assay

Human breast cancer cell lines MCF-7 and ZR75-1 were obtained from the European Collection of Cell Culture. For NOS-induction experiments, a cytokine

cocktail mixture (100 U ml^{-1} , 0.5 ng ml^{-1} interleukin-1β, 1 ng ml^{-1} interleukin-6 and 2 ng ml^{-1} tumour-necrosis factor-α) was added to the culture medium for 14–16 h. The nitrite concentration in the medium, which is used as a reflection of NO production, was determined using the Griess reaction (1% sulfanilamide, 0.1% n-1-naphthylene diamine dihydrochloride). Absorbance at 540 nm against a reference of 620 nm was measured with sodium nitrite as a standard.

Breast cancer cell lines, tissue samples and immunohistochemistry

Tissue samples of primary tumors from breast carcinoma were taken, with informed consent, from either diagnostic biopsies or after lumpectomy/mastectomy. Histopathological diagnosis was confirmed for each specimen. 15 µm sections were fixed with 4% para-formaldehyde and treated with methonal-H_2O_2. The sections were stained with a panel of antibodies: affinity-purified goat polyclonal antibody against the N-terminal peptide of Grp78 (Santa Cruz Biotechnology) diluted 1:2000; rabbit polyclonal antibody against the C-terminal peptide human iNOS (Xu *et al.*, 2000, *supra*), diluted 1:200, and rabbit polyclonal antibody against human eNOS (Alexis), diluted 1:200. The pre-immune goat and rabbit sera were used as controls. A standard Vecta-stain 'ABC kit' (Vector Laboratories) was used with indirect avidin-biotin horseradish peroxidase staining. Color was developed with diaminobenzididine (DAB). Positive staining was scored as (a) positive or negative in different cell types (b) on an arbitrary intensity scale 0 to +++ (+++ = strongest) and (c) in numbers of positive events per field or percent of section stained. Two independent qualified assessors scored less than 10% disagreement for the procedure.

Human breast cancer cell line ZR75-1 was from the European Cell Bank (ECACC). High density tissue microarrays of breast and prostate cancer samples were from Clinomics Biosciences (Pittsfield, MA). The sections were stained with anti-Grp78 scFv (1/100) or an affinity-purified goat polyclonal antibody against the N-terminal peptide of Grp78 (Santa Cruz Biotechnology) diluted 1:2000. Staining with pre-immune goat sera was used as a control. Indirect immunofluorescence was used to visualize Grp78 protein on the cell surface. Cells were grown on eight-well multi-test slides, washed with PBS, and fixed with 2% formaldehyde in PBS for 30 min at room temperature (RT). For anti-Grp78 scFv staining, a second antibody against the c-myc tag of the scFv was added (mouse mAb 9E10, Cambridge Research Biochemicals,

Wilmington, DE) followed by visualization with an anti-mouse IgG-texas-red conjugate. For the anti-peptide Grp78 antibody, cells were incubated with FITC-conjugated affinity-purified goat anti-goat IgG. After washing three times in PBS, photographs were taken using a Zeiss Axiophot fluorescence microscope and Kodak (Rochester, NY) Ektachrome film.

Selection of cell surface targeting anti-Grp78-specific scFvs

Escherichia coli strain BL21 (DE3) was transformed with the murine Grp78 expression plasmid pHis-6BiP:G565R (a kind gift from Sylvie Blond, Department of Medicinal Chemistry and Pharmacology, University of Illinois, Chicago, comprising a murine Grp78 cDNA with a C-terminal 6x His-tag , see King *et al.*, Protein Expr. Purif. 22, 148-158, 2001). Recombinant Grp78 protein was purified using a ProBond column according the manufacturers recommended conditions (Qiagen Ltd, UK). Purified Grp78 protein was used to coat plates for scFv panning experiments to isolate Grp78 specific antibody. The naïve human MRC scFv-phage libraries I + J (comprising 10^8 different scFv fragments, MRC HGMP Resource Centre) were screened using the MRC recommended protocol. In the first round of panning, 10^{12} to 10^{13} phage (in 4 ml of 2% MPBS) were incubated with 10 mg ml^{-1} recombinant Grp78. Phage recovered from this round of selection were used in a second round of panning against whole cells expressing Grp78 on the cell surface. To carry this out, EcR293 clone-11 subclone-1 cells were grown as an attached semi-confluent monolayer in 24-cm^2 flasks, and NO generated by treatment with muristerone A for 16 h (this treatment results in a 3-4 fold increase in Grp78 protein). The cells were then washed with PBS and fixed with 0.24% glutaraldehyde for 10 min at room temperature. The fixed cells were washed with PBS and blocked with DMEM/10% FCS for 1 h at room temperature. Phage from the first-round screening were precipitated with 4% PEG/0.5 M NaCl and re-suspended in water, and about 10^{13} phage in 2 ml of DMEM/10% FCS were added to $2x10^6$ EcR293 clone-11 subclone-1 cells. The culture flask was shaken gently for 2 h at room temperature and the medium removed, and the cells were washed rapidly 10 times with PBS at room temperature. Bound phage were removed by treatment with trypsin (1mg/ml in 1mM $CaCl_2$, 5-mM Tris-HCl, pH7.4) Anti-Grp78-specific scFv-phage were enriched by two further rounds of panning, against 10 μg ml^{-1} recombinant protein. After the fourth round of panning, 200 phage were picked and the resulting scFv screened using ELISA

against Grp78 protein (as described in the MRC protocol). ELISA-positive phage were used to infect *E. coli* HB2151 (a non-suppressor strain), and the resulting soluble anti-Grp78 scFv purified using Ni-NTA spin column chromatography according the manufacturers recommended conditions (Qiagen Ltd, UK).

Results

NO up-regulates Grp78 gene expression

In order to examine the role of NO on gene expression, we have used a strategy involving differential hybridization of mRNA-derived probes to normalized cDNA arrays. Two different populations of polyA$^+$ RNA were isolated, one from EcR293 clone 11 subclone-1 cells, and the other from the same cells expressing NO following treatment with 10 μM muristerone A for 24 h. 50 ng polyA+ RNA prepared from uninduced cells was labeled with the fluorescent molecule Cy 3. 50 ng polyA+ RNA prepared from induced cells was labeled with the fluorescent molecule Cy 5. The two fluorescent probe samples are simultaneously applied to a single micro array (Gem array V, Incyte Genomics, Palo Alto, California) which contains 8,372 unique annotated genes/EST clusters. Analysis of the resulting hybridization pattern shows that the gene encoding Grp78, is among the most up-regulated sequences. The uninduced signal for Grp78 is 3-fold weaker than the induced signal (Fig. 1a, wells D12, & Cy3). Similar Grp78 up-regulation was seen following hybridization to a second Gem array using poly A+ mRNA extracted from Tex293 clone-22 cells (expressing the iNOS cDNA cassette under control of the tetracycline inducible promoter, data not shown).

To support the finding that changes in hybridisation signal on the array correspond to changes in mRNA abundance, northern blotting experiments (Fig. 1b) were carried out using a probe for Grp78 (AI628509, Image clone 2286788) comprising 2.6 kb of human Grp78 cDNA. Treatment of Tex293 clone-22 cells with 10 ng/ml tetracycline (Fig 1b) significantly up-regulates Grp78 mRNA (track 2, +) compared with uninduced control cells (track 1, -). Similarly, using EcR293 clone-11 subclone-1 cells and treatment with 10 μM muristerone A, significantly up-regulates Grp78 mRNA (track 4, +) as compared with uninduced controls (track 3, -). Hybridisation with a β-actin probe as an internal control. Fig.1c shows that the relative increase of Grp78 mRNA following treatment with 10 ng tetracycline in Tex293 clone-22 cells is 2.5

(±0.2)-fold, while in EcR293 clone-11 subclone-1 cells, treatment with 1 μM muristerone A results in a Grp78 mRNA increase of 2.9 (±0.5)-fold while treatment with 10 μM muristerone A results in an increase of 4.1 (±0.8)-fold.

Western blotting with anti-Grp78 specific monoclonal antibody (Transduction Labs) shows an increase in the level of Grp78 protein (Fig. 1d) in both the tetracycline induction system (track 1 and 2) and the muristerone A induction system (tracks 3 and 4). In control experiments, an NO donor, (z)-1-[2-(2-aminoethy10-N-(2-ammonioethyl) amino]diazen-1-ium-1,2 diolate (DETA-NONOate), was also used to treat the tetracycline-regulated parental cell line-Tex293 to determine the time-course of NO-mediated Grp78 up-regulation. The NO mediated up-regulation of Grp78 was observed within 2 h DETA NONOate treatment (500 μM), and was measurable up to 20 h post-treatment (Fig. 1e).

We analysed further the relationship between NO generation and Grp78 expression using a reporter construct encoding luciferase under the control of the human Grp78 promoter. This promoter construct spans the region from −596 to +76, and includes three endoplasmic reticulum stress response elements (ERSE). In transient-transfection assays using Tex293 cells, treatment with DETA NONOate (200 μM and 500 μM), results in a 1.9-fold and a 3.3-fold increase, respectively, in the activity of the Grp78 promoter (Fig. 1f).

Grp78/Bip is a major hypoxic-response protein in the ER. In both of the iNOS cDNA-inducible cell lines (EcR293 clone-11 subclone-1, and Tex293 clone-22) there is a profound inhibition of respiration accompanying the Grp78 protein increases. For example, generation of NO results in a 60% (±20%) inhibition (n= 5) of respiration in EcR293 clone 11 subclone1 cells (16 h treatment with 10 μM muristerone A), and a 53% (±6%) inhibition (n=5) of respiration in Tex293 clone-22 cells (16 h induction with 10 ng/ml tetracycline).

It has been reported that cells failing to generate a calcium-mediated Grp78 stress response are destined to undergo cell death in response to chemical stress following treatment with the Ca^{2+} ionophore A23187 or the selective ER Ca^{2+}-ATPase inhibitor thapsigargin (TG). More recently, the vulnerability of TG-treated mouse lymphoma cells to apoptosis has been shown to be due to failure to signal a Grp78 stress response. The protective role of the NO-induced up-regulation of Grp78 was assessed using TG-induced cell toxicity experiments. EcR293 clone-11 subclone-1

cells were grown for 24 h in the presence of muristerone A to generate NO. An equal number of untreated cells were also prepared. Nitrite/nitrate concentration in the medium, was measured as a reflection of NO generation, (Fig. 2a) after overnight induction. 24 h post-induction, cells were washed with PBS and treated with TG at 37 nM for 48 h. Cell viability was determined by trypan blue staining, and by LDH analysis. Following this treatment, uninduced control cells (Fig. 2b) undergo 90% cell death, while induced cells (generating NO) are protected, and show only 22% cell death. The generation of NO in the cell appears to provide more than 60% protection against toxicity mediated by TG.

To support the hypothesis that this protection is mediated by the concomitant up-regulation of expression of Grp78, we have used Grp78-specific antisense oligonucleotides (20 μM) to attempt to down-regulate Grp78 expression in EcR293 clone11 subclone-1 cells. Cells were treated with muristerone A to generate NO, and were subsequently washed with PBS and treated with 37 ng/ml TG. Treatment with antisense oligonucleotides (Fig. 2c) results in a significant (30-40%) decrease in cell survival in the NO-generating cells. Treatment with the same concentration of control oligonucleotides (sense sequence) has no effect. To further support the finding that this protection directly involves Grp78 expression, the full-length human Grp78 cDNA sequence was cloned into the mammalian expression vector pLP-IRES2-EGFP vector under the control of the CMV/IRES promoter. A permanent cell line expressing this construct, (clone-1), expresses 3-4-fold more Grp78 protein than the empty-vector control cell line (Fig. 2d) and is 60% more resistant to TG than the control cell line.

To determine whether or not Grp78 was up-regulated in tumor samples, we hybridised a Grp78 cDNA probe to two panels of tumors; a human colon tumor panel and a breast cancer tumor panel. These tumors were selected as both classes have been shown previously to be capable of high-level expression of iNOS or eNOS. Only one sample in the colon tumor group showed up-regulation of Grp78 (data not shown), however all four of the breast cancer samples showed increased Grp78 gene expression, ranging from 1.5-fold to 6-fold compared to controls (Fig.3). Interestingly, the two invasive ductal tumors (samples 1 and 3) also showed up-regulation of iNOS (Fig.3).

The findings from the northern blot analysis were extended by immunohistochemistry using affinity–purified anti-Grp78 antibody on a panel of clinical tumor samples. Stained tissue sections from normal breast tissue (n=4)

compared with breast cancer tissues (n=19) showed that breast cancer cells express high levels of Grp78 protein compared with normal breast epithelial cells (data not shown). Positive staining was not only detectable in three different grades of invasive ductal carcinoma, but is also present in *in situ* ductal carcinomas and Lobular carcinomas.

Breast tumours are histologically and biochemically heterogenous and may contain a variety of cell types in addition to the carcinoma cells. Haematoxylin/eosin (H/E) staining showed that this invasive grade III carcinoma (Ca) also contained benign fibroadenoma (cross), and immediately surrounding proliferating stromal cells (S) in addition to infiltrating of cells of lymphoid origin (L). Strikingly, only the cytoplasm of epithelial cancer cells (Ca) was strongly stained (intensity +3), while cells within the fibro adenoma were weakly stained (intensity +1). The immediately surrounding proliferating stromal cells (S) and infiltrating cells of lymphoid origin (L) was not stained. In all 15 cases of invasive ductal carcinoma, the cancer cells showed strong staining for Grp78, while other cell types exhibit either weak or negligible staining (data not shown). These findings suggest that Grp78 is an excellent marker for breast cancer.

Grp78 antibody also reacted strongly with ducatal carcinoma *in situ*, which currently accounts for 50% of breast cancers. Interestingly, both iNOS and eNOS were also expressed strongly in these *in situ* ductal carcinomas. High expression of iNOS protein could also be seen in many of the high grade invasive carcinomas while eNOS can be detected in the surrounding myoepithial and micro vessels (data not shown).

Two breast cancer cell lines were selected for further *in vitro* studies. MCF-7 (Hamman *et al.*, Cell 92, 747-758, 1998) and ZR75-1 (Kaufman, Genes Dev. 13, 1211-1233, 1999) were treated with a cytokine cocktail for overnight induction of iNOS . The nitrite concentration in the medium (10^6 cells), was measured as a reflection of NO production, and was significantly increased (Fig. 4a). Grp78 mRNA was also found to be increased significantly in both cell lines following cytokine induction (Fig. 4b). This up-regulation of Grp78 mRNA can be partially abrogated by addition of the NOS-inhibitor L-NIO (Fig. 4c), suggesting a regulatory role for NO, although reversal is not complete. As cytokine treatment is likely to result in the activation of multiple signaling pathways, the experiments were repeated with an NO-donor, DETA NONOate. The breast cancer cell line ZR75-1 was treated with different concentrations of DETA NONOate for 24 h at concentrations of 250 μM and 500 μM DETA NONOate (Fig. 4d), resulting in 1.4-fold and 2.2-fold increase in Grp78 mRNA (Fig. 4e).

In order to investigate directly the role of Grp78 protein in breast cancer, we transfected the human breast cancer cell line MCF-7 Tet-on (Clontech) with the construct, pLPTre–Grp78, in which human Grp78 cDNA expression is placed under the control of an inducible tetracycline promoter. After co-transfection with a selection marker (pTK-hyg) a permanent cell line has been established in which Grp78 protein can be increased 5-7 following treatment with 10 ng/ml doxycycline (Fig. 4f). This cell line was used in cell survival experiments using thapsigargin (TG, 19 ng/ml), and demonstrated that doxycycline-induced cells (with increased Grp78) show 20% increased survival after 48 h compared with uninduced cells.

To determine the protective role of Grp78 in breast cancer cell survival, we have incubated two breast cancer cell lines, (MCF-7 and ZR75) with Grp78-antisense oligonucleotides and treated the cells with thapsigargin. Treatment with Grp78-antisense reduces significantly breast cancer cell viability (Fig. 4h), resulting in more than 20% increased cell death in both MCF-7 and ZR75-1 as compared with controls (untreated cells or cells treated with Grp78-sense oligonucleotides). Furthermore, immunological staining using Grp78 specific antibody demonstrates that Grp78 protein is reduced in antisense treated cell cultures (data not shown).

Targeting cell surface localized Grp78 using scFvs

Anti-Grp78 scFv were isolated following panning experiments with the MRC scFv-phage libraries against recombinant protein and whole cells as described above. Using this novel strategy, twenty-four anti-Grp78 scFv were isolated, and one, Grp78-scFv clone-19 selected for further study. A western blot analysis of Grp78 protein using scFv antibody was carried out. The purified scVF was thus used for western blot analysis of EcR293-clone-1 sublone-1 cells with (treated) or without muristerone A induction for 16 hours. Recombinant protein BSA and 6-his-tagged protein Grp78 were used as control. This confirmed that the anti Grp78-scFv clone-19 can detect Grp78 protein in western blots of whole cell extracts of EcR293 clone-11 subclone-1 cells as well as recombinant murine Grp78 protein. SDS-polyacrylamide gel electrophoresis analysis of soluble Grp78-ScFv clone 19 was also carried out. Soluble scFv was produced by induction of the Grp78-scFv clone-19 phagemid in *E. coli* HB2151, and purified by Ni-column chromatography. A 29KDa protein could be detected following purification. The purified soluble Grp78-scFv clone-19 thus has an

apparent molecular weight of around 29 kDa, consistent with the deduced amino acid sequence (see Fig. 7). The antibodies detect cell surface localized Grp78 in NO-generating EcR293 clone-11 subclone-1, and cell-surface and cytoplasmic staining in paraffin sections of human breast cancer biopsy samples.

Anti-Grp78 scFv and cell viability

We have shown that treatment of EcR293 clone-11 subclone-1 cells with muristerone A results in the NO-mediated increase in Grp78, which is responsible for protection against the selective ER Ca^{2+}-ATPase inhibitor thapsigargin. Following NO-generation, a considerable amount of the newly synthesized Grp78 is targeted to the cell surface. To determine whether anti-Grp78 scFv can reverse this protection, 10 µg of Grp78-scFv clone-19 was added to the culture medium. Control cells were treated with 10 µg of scFv-20 (a negative control scFv that does not recognize Grp78). Treatment of the cells for 48 h resulted in a significant (more than 50%) reduction in cell survival in TG-treated NO-generating cells, while treatment with the same concentration of control antibody had no significant effect on cell viability. While NO-generating cells exhibited very little apoptosis, the Grp78-scFv clone-19 treated cells showed considerable apoptosis following staining with Hoechst 33342. (see Fig. 5).

Cell viability was determined using the LDH assay (Roche, Lewes, UK), and nuclear chromatin condensation was detected using exposure to the Hoechst dye H33342 (10 µg/ml) for 15 min. Nuclei were visualized using a Leica DMIRB inverted fluorescence microscope. For each sample, a minimum of 200 cells were counted, and condensed or fragmented nuclei were expressed as a percentage of the total number of nuclei. To further test the functional significance of the scFv antibody, the breast cancer cell line ZR75-1 and prostate cancer cell line PC3 were plated (at a density of 0.5 x 10^4 cells/ml) on 35mm Petri dishes. When the cultures reached 10–20% confluence, 50 µg of purified antibody was added to the culture medium, and cells counted with a Coulter counter (Technical Communications, Coulter Electronics) at the intervals indicated in Fig. 6. All cell growth experiments were performed in triplicate. As shown in the Figure, the growth of both the breast cancer cell line ZR75-1 (A) and the prostate cancer cell line PC3 (C) is significantly retarded following treatment with the anti-Grp78 scFv-19, while the growth of the non-breast cancer luminal cell line HB3 (B) is not affected.

Discussion

In summary, we have demonstrated that NO up-regulates an important endoplasmic reticulum chaperone, Grp78. To our knowledge, this is first time NO has been shown to be involved in a major ER functional regulatory process. Grp78 functions as a molecular chaperone by associating transiently with nascent proteins as they traverse the ER and aiding in their folding and transport. In this compartment, Grp78 also functions as a Ca^{2+} storage protein. Agents such as thapsigargin, that induce calcium depletion from the ER by inhibiting ER Ca^{2+} ATPase induce Grp78 expression, which in turn, provide cytoprotective response to cytotoxic cascades involving perturbed calcium homeostasis and oxygen-radical production. Indeed, we have demonstrated here that NO-mediated Grp78 up-regulation provides significant protection to thapsigargin-mediated apoptosis. It is possible that the NO-mediated ER response and mitochondrial respiration are integrated parts of an overall response to variations in intracellular calcium that are central to the control of cellular metabolism, cell cycle, signal transduction, protein synthesis, transcription, apoptosis and cell survival. Such conditions as perturbation of protein folding and accumulation of mis-folded proteins in the ER (that induce ER stress) appear to act at an early stage of the cell death process prior to disruption of calcium homeostasis, excessive accumulation of reactive oxygen species (RO) and mitochondria dysfunction. By regulating the major ER chaperone Grp78, NO could play an important role in ER-mediated Ca^{2+} homeostasis regulation.

It is interesting to note that both inducible nitric oxide synthase and Grp78 are involved in cellular survival during chronic hypoxia. In poorly vascularised solid tumors, where glucose deprivation, chronic anoxia, and low pH are known to persist, Grp78 has been found as one of the major stress inducible proteins.

It is possible that the increase in NO may protect cancer cells in solid tumors, allowing them to survive the hypoxia, low glucose and disruption of calcium homeostasis, which is frequently observed in breast cancer development. NO can increase Grp78 via a mechanism involving regulated intramembrane proteolysis (RIP). The increase in concentration of NO in a cell leads to the flux of Ca^{2+} through the mitochondrion which in turn activates the membrane-bound protease SIP (site-1 protein). SIP cuts the membrane bound transcription factor ATF6, resulting in the

production of a soluble (pSOATF6) form of the transcription factor that migrates to the nucleus and upregulates Grp78 expression

In cancer, up-regulation of Grp78 has been shown to result in increased resistance to cell-mediated lysis by cytotoxic T lymphocytes (CTLs) and tumor necrosis factor (TNF), while cells unable to induce Grp78 cannot form tumors. In addition, induction of Grp78 expression correlates with the development of resistance to anti-tumor drugs. Recent experiments have reported that Grp78 can be found on the surface of cells, including tumour cells, and a Grp78-binding peptide has been used for the delivery of a cytotoxic peptide sequence both *in vitro* and *in vivo*. The surface localization of Grp78 may make it an ideal target as a diagnostic marker in cancer screening. Surprisingly, the Grp78-scFv clone-19 antibody described here is not only able to identify surface-localized Grp78, but is also able to reverse the cytoprotective properties of Grp78. While treatment with Grp78-scFv clone-19 can result in apoptosis, the cell death effect is more pronounced in combination with TG. Interestingly, modified TG analogues coupled to a peptide carrier that is a substrate for the prostate-specific antigen (PSA) are under evaluation as a treatment for prostate cancer. Overall it is possible that the Grp78-scFv clone-19 antibody described here make a novel therapeutic approach possible for the treatment of cancers (perhaps in combination with TG analogues) where Grp78 is over-expressed.

CLAIMS

1. A method for identifying a substance which inhibits or prevents resistance to a chemotherapeutic agent or a radiotherapeutic agent and/or which may be used in the treatment of a cancer, which method comprises determining whether a test substance is an inhibitor of Glucose-regulated protein 78 (Grp78).

2. A method according to claim 1, comprising determining whether the test substance is an inhibitor of Grp78 expression and/or activity.

3. A method according to claim 1 or 2, wherein determining whether a test substance is an inhibitor of Grp78 comprises:

(i) providing a population of cells which is capable of generating NO;

(ii) contacting the population of cells with thapsigargin or an analog thereof, under conditions that lead to the generation of NO by the cell population, in the presence of the test substance; and

(iii) determining the proportion of the cell population which dies thereby to determine whether the test substance is capable of inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

4. A method according to claim 3, wherein cells of the NO population capable of generating NO contain a polynucleotide construct, which construct comprises:

(a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof.

5. A method according to claim 2, wherein determining whether the test substance is an inhibitor of Grp78 expression comprises:

(i) providing a cell containing a polynucleotide construct, which polynucleotide construct comprises a Grp78 promoter, or a functional variant thereof, operably linked to a coding sequence;

(ii) contacting the cell with the test substance under conditions which in the absence of the test substance would lead to expression of the coding sequence; and

(iii) determining whether the test substance inhibits expression of the test substance thereby to determine whether the test substance is an inhibitor of Grp78 expression.

6. A method according to claim 2, wherein determining whether the test substance is an inhibitor of Grp78 activity comprises determining whether or not the test substance is capable of binding to Grp78.

7. A method according to claim 2 or 6, wherein determining whether the test substance is an inhibitor of Grp78 activity comprises:

(i) providing a Grp78 or a functional variant thereof;

(ii) contacting the Grp78 with the test substance under conditions that in the absence of the test substance would lead to activity of the Grp78 or functional variant; and

(iii) determining whether the test substance inhibits activity of the Grp78 or functional variant thereby to determine whether the test substance is an inhibitor of Grp78 activity.

8. A method according to any one of claims 2 to 7, wherein the test substance is an antibody.

9. A method according to claim 8, wherein the antibody is a single-chain antibody variable fragment (scFv)

10. An inhibitor of Grp78 for use in a method of treatment of the human or animal body by therapy.

11. An inhibitor according to claim 10 for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

12. An inhibitor according to claim 10 or 11 for use in the treatment of a cancer.

13. An inhibitor according to any one of claims 10 to 12 which is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

14. Use of an inhibitor of Grp78 in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

15. Use of an inhibitor of a NOS in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

16. Use of an inhibitor of a Grp78 in the manufacture of a medicament for use in the treatment of a cancer.

17. Use according to any one of claims 14 to 16 wherein the inhibitor is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

18. A method of treating or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host, which method comprises the step of administering to the host an effective amount of an inhibitor of Grp78 or an inhibitor of a NOS.

19. A method of treating a cancer in a host, which method comprises the step of administering to the host an effective amount of an inhibitor of Grp78.

20. A method according to claim 18 or 19, wherein the inhibitor is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

21. A substance identified by a method according to any one of claims 1 to 9.

22. A substance according to claim 21 for use in a method of treatment of the human or animal body by therapy.

23. A substance according to claim 22 for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

24. A substance according to claim 23 for use in the treatment of a cancer.

25. A substance according to any one of claims 22 to 24 which is inhibitor is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

26. Use of a substance according to claim 21 in the manufacture of a medicament for use in inhibiting or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent.

27. Use of a substance according to claim 21 in the manufacture of a medicament for use in the treatment of a cancer.

28. Use according to claim 26 or 27, wherein the substance is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

29. A method of treating or preventing resistance to a chemotherapeutic agent or a radiotherapeutic agent in a host, which method comprises the step of administering to the host an effective amount of a substance according to claim 21.

30. A method of a cancer in a host, which method comprises the step of administering to the host an effective amount of a substance according to claim 21.

31. A method according to claim 29 or 20, wherein the substance is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

32. Products containing an inhibitor of a NOS and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer.

33. Products containing an inhibitor of Grp78 and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer.

34. Products containing a substance according to claim 21 and a chemotherapeutic agent or a radiotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a cancer.

35. Products according to any one of claims 32 to 34, wherein the substance is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

36. Use of an inhibitor of a NOS in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer.

37. Use of an inhibitor of Grp78 in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer.

38. Use of a substance according to claim 21 in the manufacture of a medicament for use with a chemotherapeutic agent or a radiotherapeutic agent in the treatment of a cancer.

39. Use according to any one of claims 36 to 38, wherein the substance is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

40. A method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of an inhibitor of NOS and a chemotherapeutic agent or a radiotherapeutic agent.

41. A method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of an inhibitor of Grp78 and a chemotherapeutic agent or a radiotherapeutic agent.

42. A method of treating a host suffering from a cancer, which method comprises the step of administering to the host effective amounts of a substance according to claim 21 and a chemotherapeutic agent or a radiotherapeutic agent.

43. A method according to any one of claims 40 to 42, wherein the substance is an scFv having the amino acid sequence set out in SEQ ID NO: 2 or a functional variant thereof.

44. A method for determining the presence of a cancer in a subject, which method comprises typing the gene encoding Grp78 in the subject.

45. A method according to claim 44, wherein the typing comprises determining the amount of expression of the gene encoding Grp78 in the subject.

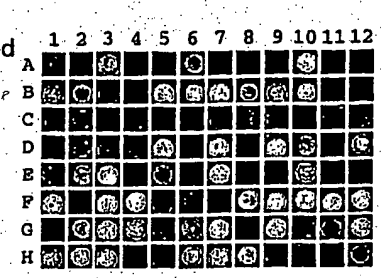
46. A method according to claim 45, wherein increased expression of the gene encoding Grp78 in a tissue of the subject, as compared to the amount of expression of that gene in a subject which does not have a cancer, or as compared to non-cancerous tissue of the subject, indicates the presence of a cancer in the subject.

47. A method according to any one of claims 44 to 46, wherein the typing is carried out by use of an antibody.

48. A method according to claim 47 wherein the antibody is an scFv having the amino acid sequence of SEQ ID NO: 2.

49. An scFv having the sequence set out in SEQ ID NO: 2 or a functional variant thereof.

a. uninduced



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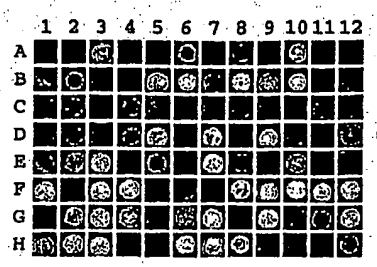
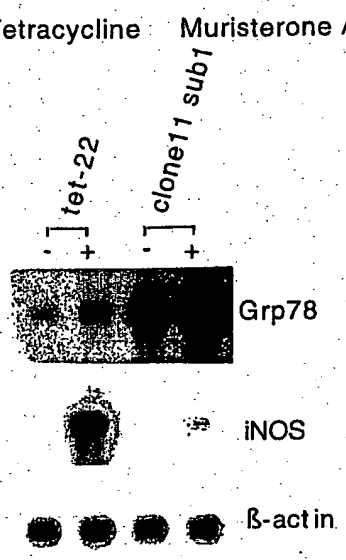


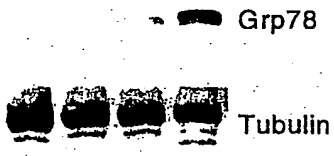
mRNA blots

b.

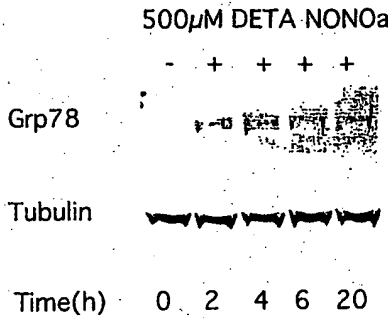


Protein blots

d.



e.



c.

Cell Lines	Relative fold induction grp78 mRNA	NO_2^- μM/ 10^6cells
Tex clone22		
uninduced	1	0.5
10ng Tc	2.5±0.2	95±10
EcR293		
Clone11 sub-1		
uninduced	1	0.2
1μM induced	2.95±5	10±3
10μM induced	4.1±0.8	45±7

f.

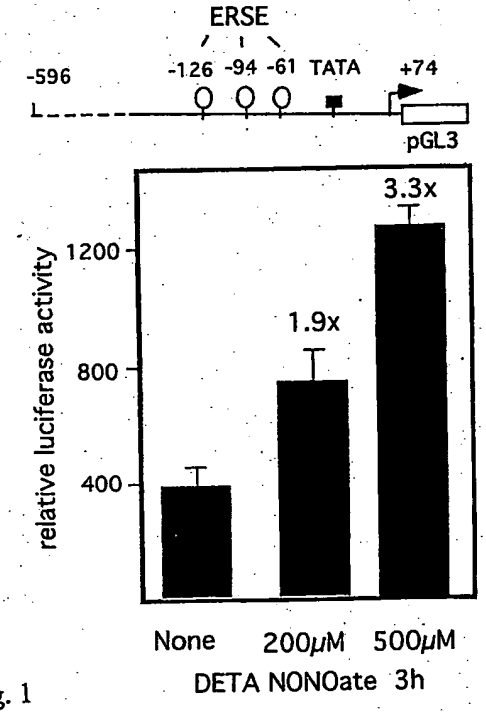


Fig. 1

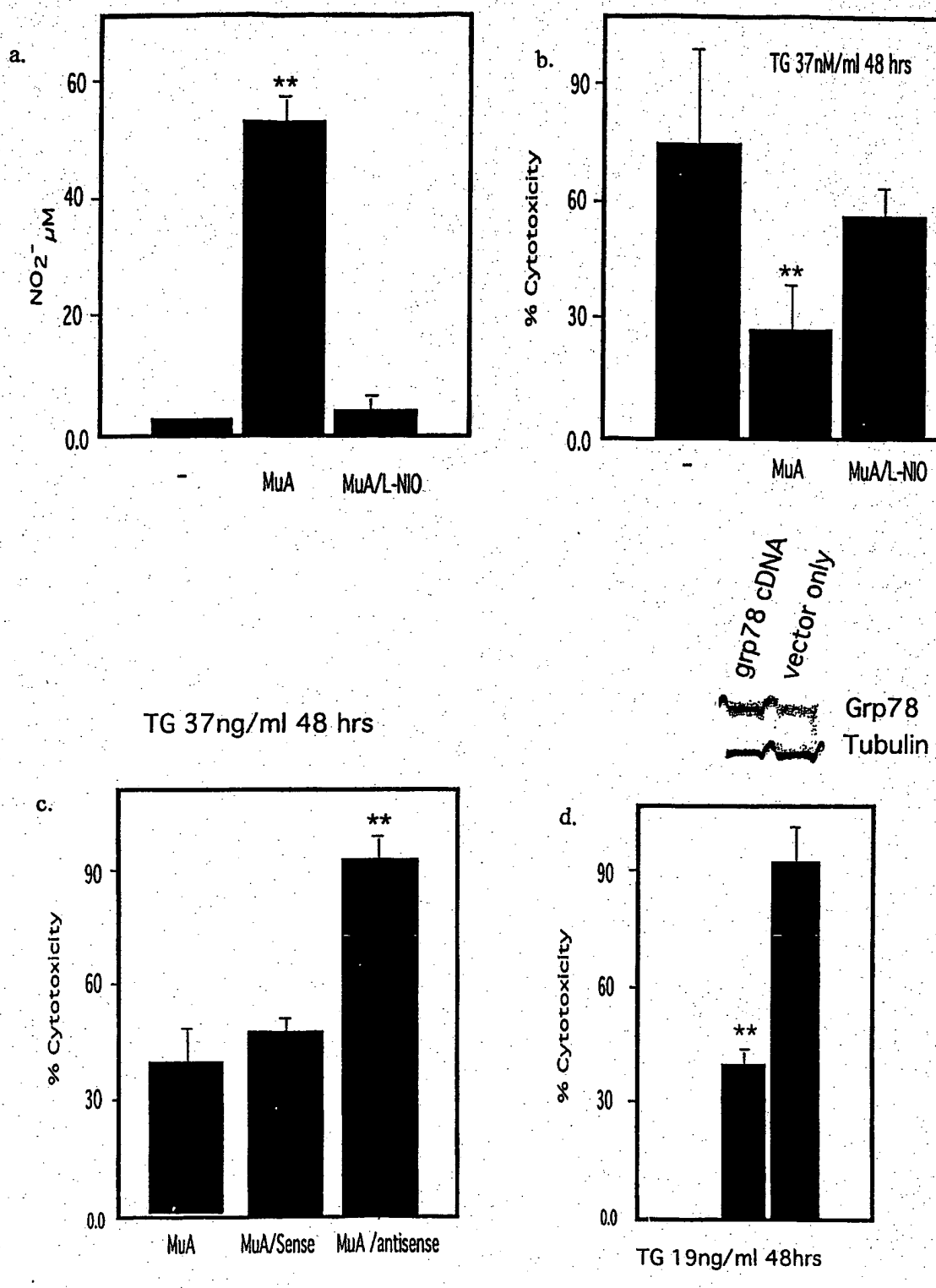
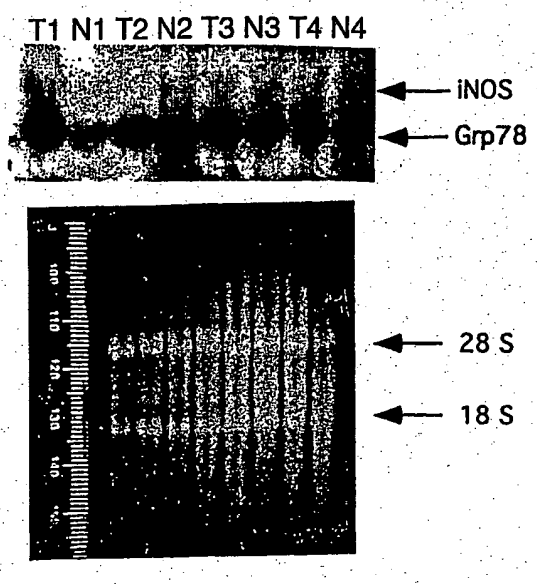

a.
60
40
20
0.0
NO_2^- μM
**
-
MuA
MuA/L-NIO
b.
TG 37nM/ml 48 hrs
90
60
30
0.0
% Cytotoxicity
**
-
MuA
MuA/L-NIO
TG 37ng/ml 48 hrs
grp78 cDNA
vector only
Grp78
Tubulin
c.
90
60
30
0.0
% Cytotoxicity
**
MuA
MuA/Sense
MuA /antisense
d.
90
60
30
0.0
% Cytotoxicity
**
TG 19ng/ml 48hrs

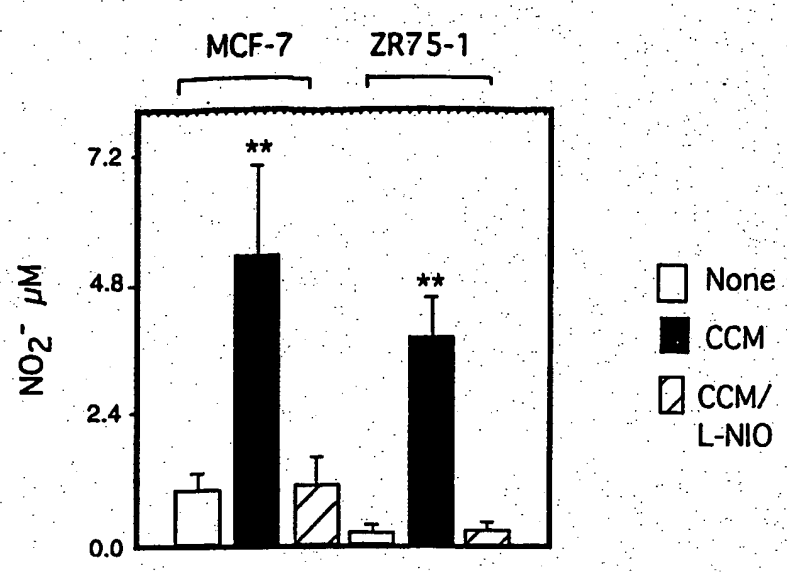
Fig. 2



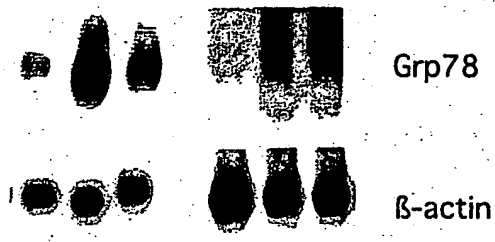
	mRNA fold increase	
	grp78	iNOS
1 Invasive Ductal / normal	5.2	2.5
2 Ductolobular / normal	2.5	0.58
3 Invasive Ductal / normal	2.8	1.3
4 Invasive Ductal / normal	1.3	0.65

Fig. 3

a.



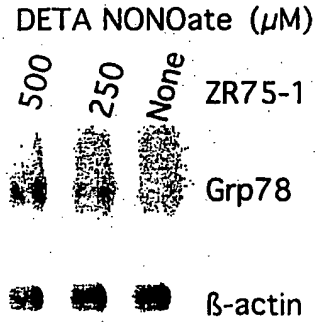
b.



c.

	MCF-7			ZR75-1		
	None	CCM	CCM /L-NIO	None	CCM	CCM /L-NIO
Relative fold induction Grp78 mRNA	1	2.1±0.5	1.5±0.3	1	3.4±0.6	2.5±0.5

d.



e.

ZR75-1	DETA NONOate(μM)		
	None	250	500
Relative fold induction Grp78 mRNA	1	1.4	2.2

Fig. 4

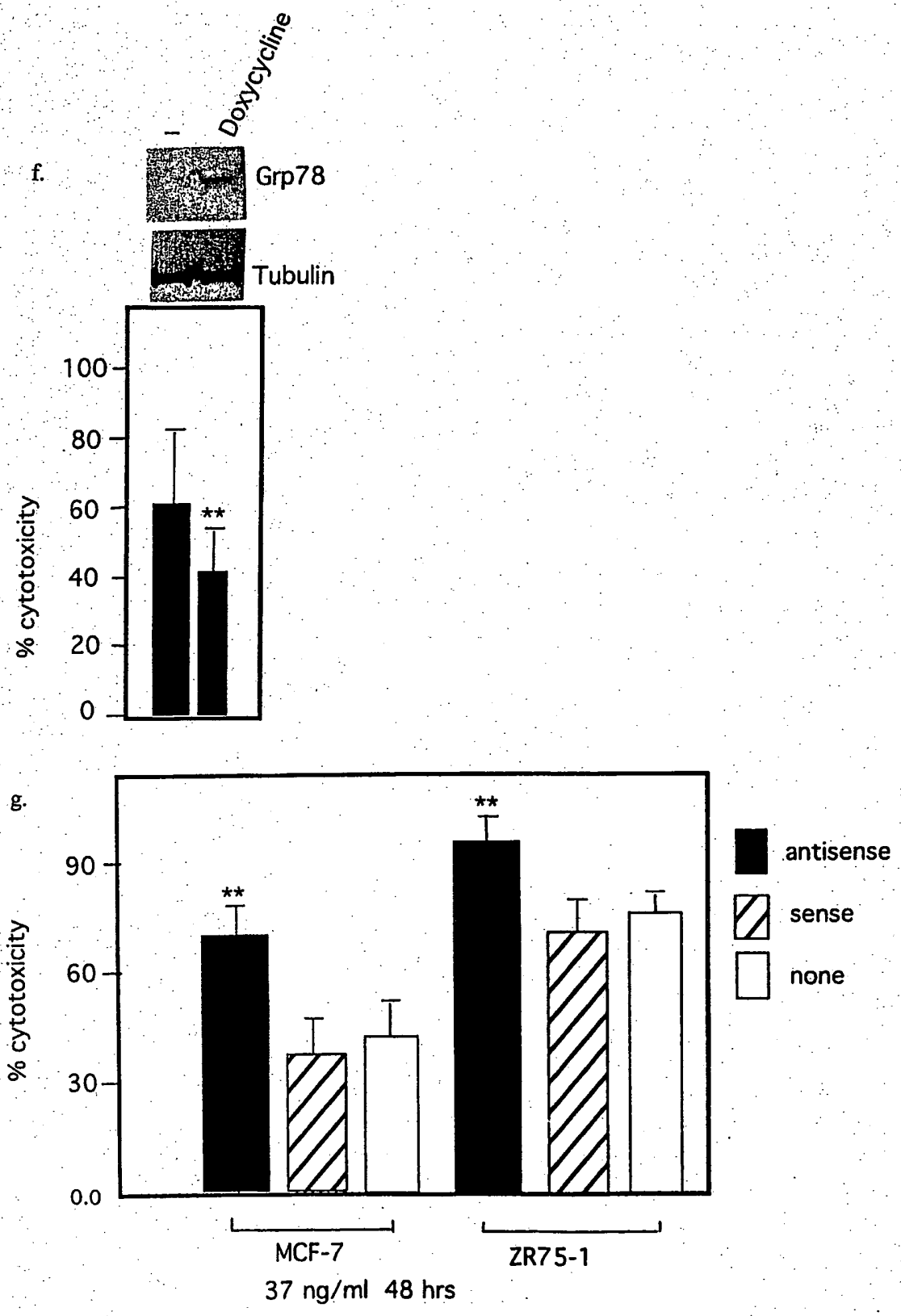
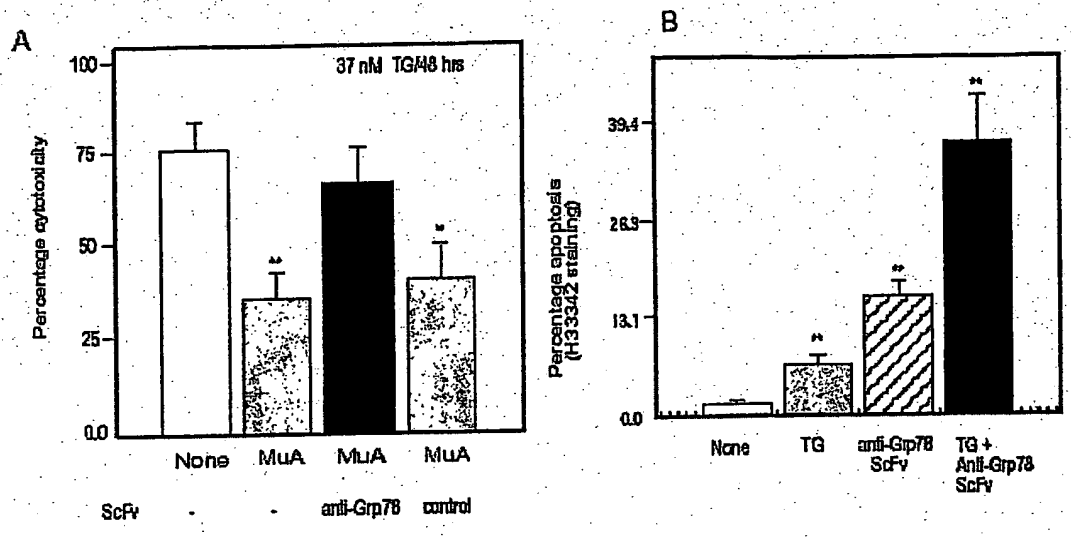


Fig. 4 (continued)



Clone 11 PC3

Fig. 5

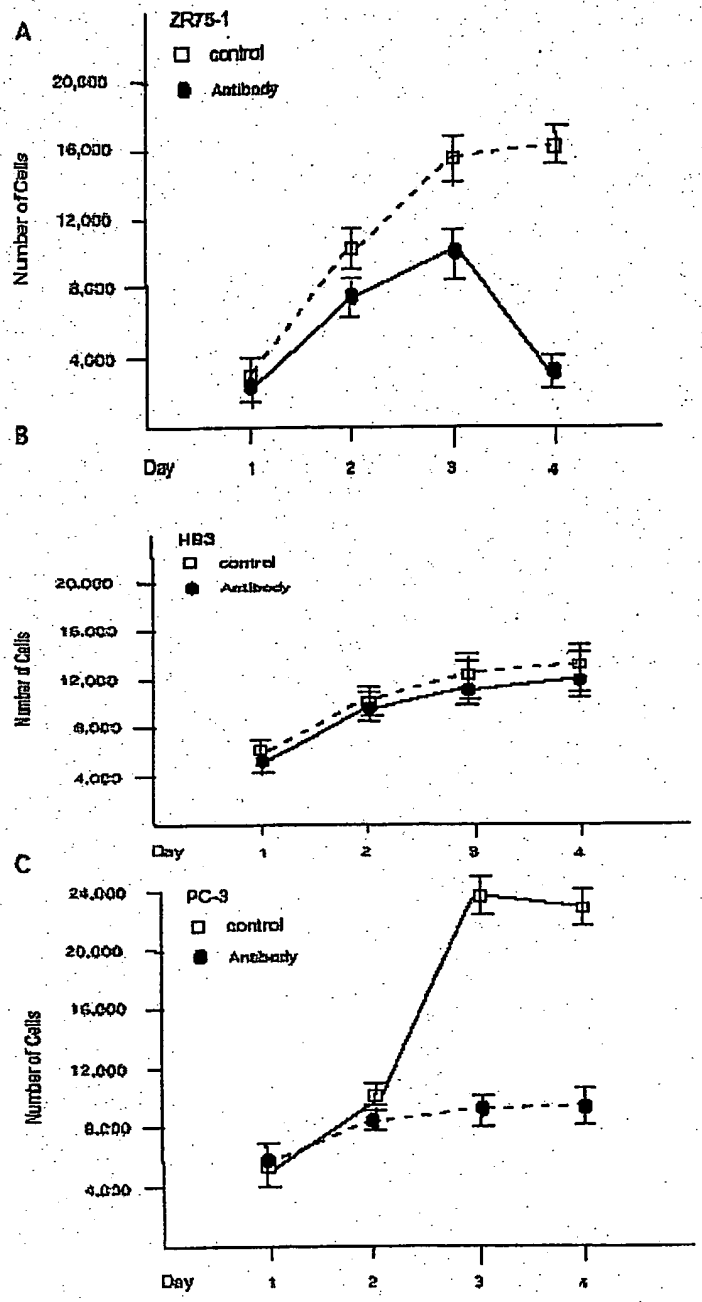

A
ZR75-1
control
Antibody
20,000
16,000
12,000
8,000
4,000
Number of Cells
B
Day
1
2
3
4
HBS
control
Antibody
20,000
16,000
12,000
8,000
4,000
Number of Cells
Day
1
2
3
4
C
PC-3
control
Antibody
24,000
20,000
16,000
12,000
8,000
4,000
Number of Cells
Day
1
2
3
4

Fig. 6

```
        E  V  Q  L  V  E  S  G  G  G  V  V  Q  P  G  R  S
       GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG TCC
                 9         18         27         36         45

                                                CDR H1
L  R  L  S  C  A  A  S  G  F  T  F  S  S  Y  G  M  H  W
CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT GGC ATG CAC TGG
       60         69         78         87         96        105

                                                CDR H2
V  R  Q  A  P  G  K  G  L  E  W  V  A  V  I  S  Y  D  G
GTC CGC CAG GCT CCA GGC AAA GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA
       117        126        135        144        153        162

S  N  K  Y  Y  A  D  S  V  K  G  R  F  T  I  S  R  D  N
AGT AAT AAA TAC TAT GCA GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT
       174        183        192        201        210        219

S  K  N  T  L  Y  L  E  M  N  S  L  R  A  E  D  T  A  V
TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCC GTG
       231        240        249        258        267        276

                                CDR H3
Y  Y  C  A  R  K  I  N  S  T  K  E  V  W  G  Q  G  T  L
TAT TAC TGT GCA AGA AAG ATT AAT AGT ACG AAG GAG GTG TGG GGC CAA GGT ACC CTG
       288        297        306        315        324        333

V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  S  A
GTC ACC GTC TCG AGT GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT AGT GCA
       345        354        363        372        381        390

L  Q  S  V  L  T  Q  P  P  S  A  S  G  T  P  G  Q  R  V
CTT CAG TCT GTG CTG ACT CAG CCA CCC TCA GCG TCT GGG ACC CCC GGG CAG AGG GTC
       402        411        420        429        438        447

                          CDR L1
T  I  S  C  S  G  S  S  S  N  I  G  S  N  Y  V  Y  W  Y
ACC ATC TCT TGT TCT GGA AGC AGC TCC AAC ATC GGA AGT AAT TAT GTA TAC TGG TAC
       459        468        477        486        495        504

                                                CDR L2
Q  Q  L  P  G  T  A  P  K  L  L  I  Y  R  N  N  Q  R  P
CAG CAG CTC CCA GGA ACG GCC CCC AAA CTC CTC ATC TAT AGG AAT AAT CAG CGG CCC
       516        525        534        543        552        561

S  G  V  P  D  R  F  S  G  S  K  S  G  T  S  A  S  L  A
TCA GGG GTC CCT GAC CGA TTC TCT GGC TCC AAG TCT GGC ACC TCA GCC TCC CTG GCC
       573        582        591        600        609        618

                                                CDR L3
I  S  G  L  R  S  E  D  E  A  D  Y  Y  C  A  A  W  D  D
ATC AGT GGG CTC CGG TCC GAG GAT GAG GCT GAT TAT TAC TGT GCA GCA TGG GAT GAC
       630        639        648        657        666        675

S  L  N  P  L  V  V  F  G  G  G  T  K  L  T  V  L  G  A
AGC CTG AAT CCT CTT GTT GTA TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT GCG
```

Fig. 7

```
          687       696       705       714       723       732

A  A  H  H  H  H  H  H  G  A  A  E  Q  N
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Fig. 7 (continued)

SEQUENCE LISTING

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<120> SCREEN FOR THE IDENTIFICATION OF AGENTS USEFUL IN THE TREATMENT OF CANCERS

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<150> GB 0404936.7

<151> 2004-03-04

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65                  70                  75                  80

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Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

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Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
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Gly Ser Ala Leu Gln Ser Val Leu Thr Gln Pro Pro Ser Ala Ser Gly
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acc ccc ggg cag agg gtc acc atc tct tgt tct gga agc agc tcc aac      480
Thr Pro Gly Gln Arg Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn
145                 150                 155                 160

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Ile Gly Ser Asn Tyr Val Tyr Trp Tyr Gln Gln Leu Pro Gly Thr Ala
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ccc aaa ctc ctc atc tat agg aat aat cag cgg ccc tca ggg gtc cct      576
Pro Lys Leu Leu Ile Tyr Arg Asn Asn Gln Arg Pro Ser Gly Val Pro
            180                 185                 190

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                85                  90                  95

Ala Arg Lys Ile Asn Ser Thr Lys Glu Val Trp Gly Gln Gly Thr Leu
            100                 105                 110

Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
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Gly Ser Ala Leu Gln Ser Val Leu Thr Gln Pro Pro Ser Ala Ser Gly
    130                 135                 140

Thr Pro Gly Gln Arg Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn
145                 150                 155                 160

Ile Gly Ser Asn Tyr Val Tyr Trp Tyr Gln Gln Leu Pro Gly Thr Ala
                165                 170                 175

Pro Lys Leu Leu Ile Tyr Arg Asn Asn Gln Arg Pro Ser Gly Val Pro
            180                 185                 190

Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu Ala Ile
        195                 200                 205

Ser Gly Leu Arg Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Ala Ala Trp
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Asp Asp Ser Leu Asn Pro Leu Val Val Phe Gly Gly Gly Thr Lys Leu
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Glu Gln Asn
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/000839

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAMORA C ET AL: "INHIBITION OF TUMOR PROGRESSION BY SUPPRESSION OF STRESS PROTEIN GRP78/BIP INDUCTION IN FIBROSARCOMA B/C10ME" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 93, July 1996 (1996-07), pages 7690-7694, XP002066166 ISSN: 0027-8424	1-3, 7-14, 16-31, 38,39, 42,43
Y	abstract page 7692, column 1, paragraph 1 page 7690, column 2, paragraph 1	4-6

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2005	20/07/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bigot-Maucher, C

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/000839

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 382 615 A (XERION PHARMACEUTICALS AG) 21 January 2004 (2004-01-21)	1-3, 7-10,12, 13,16, 17, 19-25, 27,28, 30,31, 38,39,49
Y	abstract page 6, lines 13-18; claims 1,2,14	4-6
X	GAZIT G ET AL: "DE-REGULATION OF GRP STRESS PROTEIN EXPRESSION IN HUMAN BREAST CANCER CELL LINES" BREAST CANCER RESEARCH AND TREATMENT, NIJHOFF, BOSTON, US, vol. 54, no. 2, March 1999 (1999-03), pages 135-146, XP009048575 ISSN: 0167-6806 abstract page 135, column 1, paragraph 2 - column 2, paragraph 2	1-3
X	KOONG A C ET AL: "Increased cytotoxicity of chronic hypoxic cells by molecular inhibition of GRP78 induction." INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY, BIOLOGY, PHYSICS. 1 FEB 1994, vol. 28, no. 3, 1 February 1994 (1994-02-01), pages 661-666, XP009049071 ISSN: 0360-3016 abstract page 665, column 2, paragraph 2	1,2, 10-12, 14,16, 18,19, 22-27, 29-31, 33-35, 37-39, 41-43
X	SONG M S ET AL: "Induction of glucose-regulated protein 78 by chronic hypoxia in human gastric tumor cells through a protein kinase C-epsilon/ERK/AP-1 signaling cascade." CANCER RESEARCH. 15 NOV 2001, vol. 61, no. 22, 15 November 2001 (2001-11-15), pages 8322-8330, XP001206828 ISSN: 0008-5472 abstract figure 2	1,2,7, 44-48

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/000839

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU W ET AL: "NITRIC OXIDE UPREGULATES EXPRESSION OF DNA-PKCS TO PROTECT CELLS FROM DNA-DAMAGING ANTI-TUMOUR AGENTS" NATURE CELL BIOLOGY, MACMILLAN PUBLISHERS, GB, vol. 2, no. 6, June 2000 (2000-06), pages 339-345, XP009010951 ISSN: 1465-7392 abstract page 344, column 1, last paragraph - column 2, paragraph 1; figure 1	4-6,15, 18,32, 36,40
Y	XU W ET AL: "MICROENCAPSULATED INOS-EXPRESSING CELLS CAUSE TUMOR SUPPRESSION IN MICE" THE FASEB JOURNAL, vol. 16, no. 2, February 2002 (2002-02), pages 213-215, XP009048560 abstract page 213, column 2, paragraph 4	4-6
P,X	BRENNAN P A ET AL: "Hunterian Lecture. Regulation of hypoxia-inducible factor 1 (HIF-1) by nitric oxide in oral squamous cell carcinoma." ANNALS OF THE ROYAL COLLEGE OF SURGEONS OF ENGLAND. MAY 2005, vol. 87, no. 3, May 2005 (2005-05), pages 153-158, XP009048764 ISSN: 0035-8843	15,18
Y	abstract page 154, column 2, paragraph 2 page 155, column 2, paragraphs 2,3	4-6
P,X	GIL'IANO N IA ET AL: "'Modification of cellular radiosensitivity by NO-synthase inhibitors!" RADIATSIONNAIA BIOLOGIIA, RADIOECOLOGIIA / ROSSIISKAIA AKADEMIIA NAUK. 2005 JAN-FEB, vol. 45, no. 1, January 2005 (2005-01), pages 63-67, XP009049643 ISSN: 0869-8031 abstract	32,36,40
P,X	CHOO SOO-JIN ET AL: "Deoxyverrucosidin, a novel GRP78/BiP down-regulator, produced by Penicillium sp." THE JOURNAL OF ANTIBIOTICS. MAR 2005, vol. 58, no. 3, March 2005 (2005-03), pages 210-213, XP009049106 ISSN: 0021-8820 the whole document	1-49

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/000839

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	UMEDA YUKIKO ET AL: "Prunustatin A, a novel GRP78 molecular chaperone down-regulator isolated from Streptomyces violaceoniger." THE JOURNAL OF ANTIBIOTICS. MAR 2005, vol. 58, no. 3, March 2005 (2005-03), pages 206-209, XP009049105 ISSN: 0021-8820 the whole document	1-49

INTERNATIONAL SEARCH REPORT

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PCT/GB2005/000839

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-9, 18-20, 29-31 and 40-48 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB2005/000839

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1382615 A	21-01-2004	EP 1382615 A1	21-01-2004
		AU 2003250065 A1	02-02-2004
		AU 2003250956 A1	02-02-2004
		CA 2492559 A1	22-01-2004
		WO 2004007717 A1	22-01-2004
		WO 2004007550 A1	22-01-2004